A STUDY OF SOFT-ROT IN HARDWOODS

by

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This thesis contains no material which has been accepted for any other degree or diploma in any university, and to the best of my knowledge, contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of the thesis.

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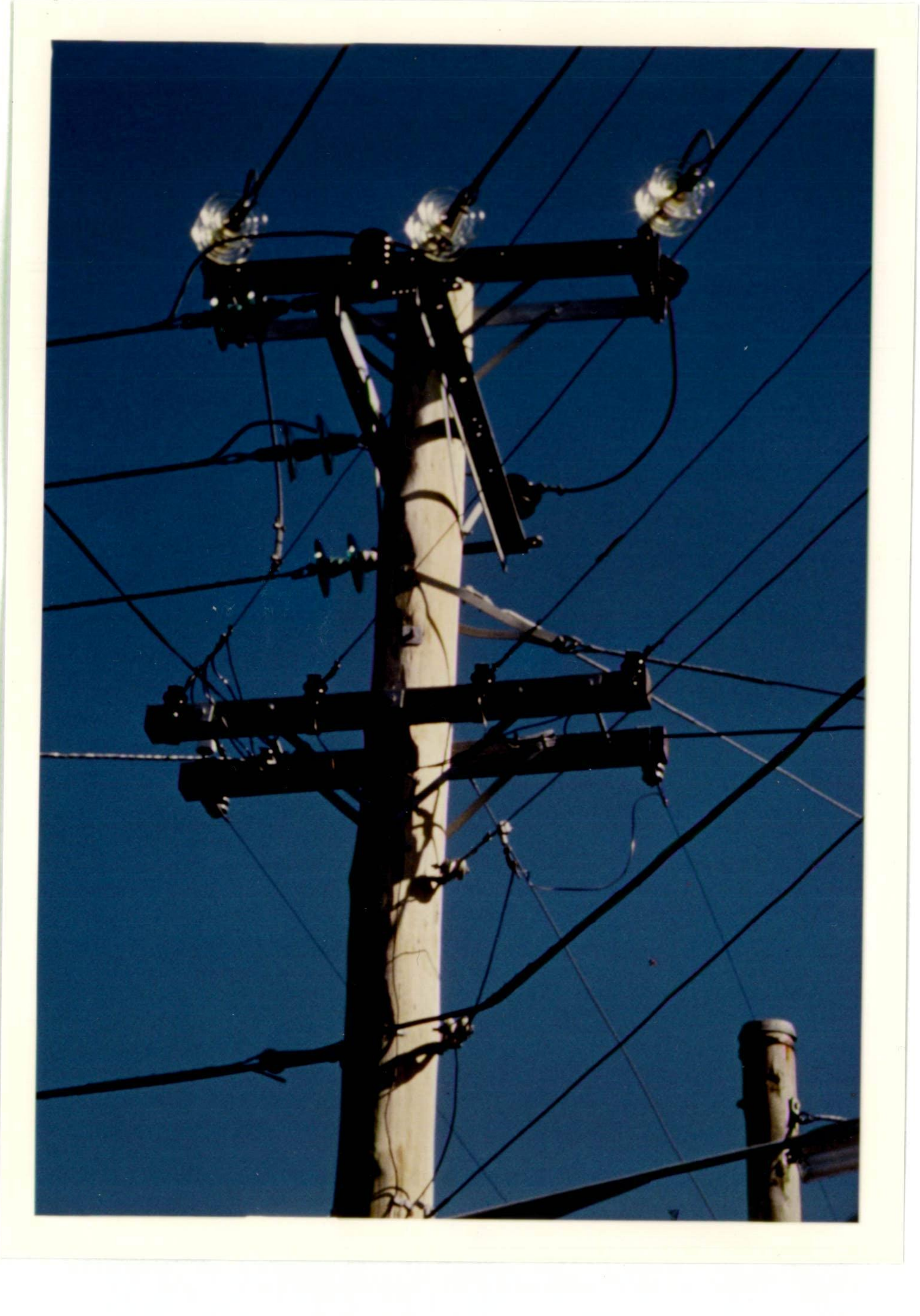

PUBLICATIONS

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ABSTRACT

The main aims of this study were to examine the wood substrate degrading capacities of microorganisms isolated from soft-rotted CCA-treated *Eucalyptus* sp. power transmission poles in ground contact, and to evaluate techniques for assessment of the degree of wood degradation caused by microbial attack. The performances of various wood preservatives, including remedial and ground-line maintenance treatment systems for poles, were assessed.

The predominant organism isolated from Tasmanian woods in this study, the Hyphomycete *Phialophora mutabilis*, had demonstrable cellulase, hemicellulase, amylase and pectic enzyme activities as well as a measurable wood-degrading capacity. *Trichoderma viride* was a highly cellulolytic fungus in pure culture, and a dominant early coloniser of untreated *E. obliqua* wood in ground contact. However, it produced minimal wood degradation in this examination.

A small ($\leq 10^4$ cells/g wood sawdust) bacterial microflora was found in untreated and preservative-treated hardwoods. Bacterial isolates possessed a number of wood-degrading enzymes. A strain of *Bacillus megaterium* (S9NC) isolated in this study, showed cellulase, xylanase, amylase and pectic enzyme activities, whilst *Cellulomonas* sp. 8N produced at least one cellulase, xylanase, pectic enzyme and laccase. However, no definite degradation of intact *E. obliqua* sapwood cell walls by pure cultures of bacteria was observed by light microscopy and scanning electron microscopy.

Fungi (including Basidiomycetes) and bacteria were early colonizers of untreated *E. obliqua* stakes in the ground. A

mutualistic relationship, albeit not a close one, was demonstrated between propagule counts of fungi and bacteria isolated from CCA-treated Eucalyptus sp. sawdusts.

Two techniques for estimating microbial activity in wood samples were developed in this study, and compared with other methods for estimating the degree of wood degradation. The methods were:

i. a Cx-cellulase assay

ii. a modified and improved chitin assay for estimation of fungal biomass in wood. Appropriate parameters for both assay methods were determined.

The Cx-cellulase and chitin assays were relatively quick and sensitive methods for assessing the degree of microbial attack of woods. These assay procedures, plus impaction determinations using the Pilodyne(R) instrument, and to a much lesser extent the fungal propagule count, were largely objective procedures. Visual and microscopic estimates of wood degradation were found to be highly subjective in this study.

Several field trials of wood preservatives were used to compare the newly developed assay methods with fungal propagule counts, Pilodyne(R) impaction determinations, and visual and microscopic estimates of wood degradation. The trials examined were remedial treatments of transmission poles at Warrane, Tasmania, and bandage treatments of poles at Grafton, N.S.W. and Coff's Harbour, N.S.W. In addition, an E. obliqua preservative-treated sapwood stake trial was emplaced at Grove, Tasmania.

In this study, the best performed remedial preservative systems were the Wolman CFB bandage and the CSIRO-developed

Busan 30 and Blue 7 (Mark IV) bandages. These bandage systems show promise as agents for treatment of soft-rot attack in Australian power transmission poles.

(R) = Registered trademark.

INTRODUCTION

Sapwood decay at the ground-line of preservative-treated *Eucalyptus* spp. power transmission poles is of particular concern to Australian electricity supply authorities. The problem is aggravated by a declining supply of durable eucalypt timber species of the girth needed for such poles. Further, the attack occurs at the ground-line where the major portion of the wood bending stress is borne by the annular sapwood ring (Tamblyn and Dale, 1963).

This decay or soft-rot degradation, is caused by members of Fungi Imperfecti and Ascomycetes (lower fungi) and is prevalent in woods where growth of Basidiomycetes is retarded, as, for example, in CCA-treated sapwood. Soft-rot degraded wood has a distinctive appearance, it is soft, and often breaks with a distinct carrotty fracture (Greaves, 1977a).

In Queensland alone, it has been estimated that 300,000-400,000 pressure impregnated hardwood transmission poles were affected by soft-rot in 1977 (Greaves, 1977a). Of the population of 150,000 poles in Tasmania, 19,000 were thought to be badly affected by soft-rot (Shaw, 1978). The problem, however, is not economically serious in this State as yet, as very few poles have been condemned specifically because of soft-rot decay (Nahodill, 1982, pers. comm.). This could shortly change, since the average cost of replacing degraded poles is now $450-700 depending on location, and this will obviously increase in the future with inflation.

Whilst the long-term aim of devising improved preservative formulations to treat sapwood is being pursued by some research authorities, remedial wood preservatives are needed to extend the life of poles in service. The C.S.I.R.O. Division of Building Research (Melbourne, Australia) has developed various toxicant-containing bandages for the ground-line treatment of soft-rot attack of poles. Some of these preservative systems were included in a Tasmanian field trial and examined as part of this study.

LITERATURE REVIEW

LITERATURE REVIEW

1.1 GENERAL

1.1.1 Wood Chemistry

Celluloses, hemicelluloses and lignin are the three main constituents of the cell walls of wood xylem. Cellulose is a linear polymer based on anhydroglucose units coupled by β-1,4-glucosidic bonds. It tends to form elementary fibrils in which the polymer chains are orientated in parallel and interlocked with hydrogen bonds (Frey-Wyssling, 1937; Mandels and Reese, 1965; Mühlethaler, 1967; Sihtola and Neimo, 1975). These elementary fibrils of 35 A^{o} approximate diameter are the smallest structural units of the microfibrils and large fibres (Preston, 1965). In wood, the individual fibres contain a central core of crystalline cellulose, interrupted at irregular lengths by regions of non-cellulosic polysaccharides (Preston, 1979).

Hemicelluloses are polysaccharides of low molecular weight that normally occur in plant material together with cellulose. These are extractable by aqueous alkali (Schulze, 1892; Timell, 1964). There are conflicting reports as to whether these substances can be readily imaged in the electron microscope as separate, identifiable fibrillar material (Coté, 1977; Kerr and Goring, 1977). The dominant hemicelluloses in hardwoods are xylans, based on xylose sugar residues, whilst softwoods contain glucomannans (mannose residues) as the major hemicellulose component (Meier, 1964; Takahashi and Nishimoto, 1973).

The third major component of wood is lignin, a very complex material composed of methoxylated phenyl propane units (Kaplan and Hartenstein, 1980). As lignin is resistant to chemical and biological degradation, its close association with celluloses and hemicelluloses in the plant cell wall may hinder the biodegradation of the walls (Higuchi, 1971; Polcin and Bezúch, 1977; Reddy and Forney, 1978; Dekker and Lindner, 1979). Literature pertaining to the structure and chemistry of lignin has been comprehensively reviewed by Adler (1977).

Wood extractives (predominantly polyphenols, tannins, tropolones and fatty acids) are found in comparatively small quantities in woods (Dadswell and Hillis, 1962). The concentration of these substances is usually greatest in the heartwood (Coté, 1977). Other substances present in wood may include pectins, starches (both predominantly found in sapwood) and possibly structural protein (Preston and Wardrop, 1949; Thornber and Northcote, 1961; Mühlethaler, 1967).

1.1.2 Wood Structure

An account of the relationship between the growth of a tree and the subsequent timber produced has been given by Williams (1953), whilst *inter alia* Jane (1956), Dadswell and Hillis (1962), Esau (1965) and Desch (1968) have reviewed the literature pertaining to wood structure.

The wood of both gymnosperms (naked seeds) and angiosperms (enclosed seeds) is composed of xylem tissue for water transport and structural support, and phloem or bark tissue for food transport. The sapwood (living cell material) and the dead heartwood of a tree are composed of xylem tissue of varying degrees of lignification and occlusion.

The softwoods (coniferous species) are located mainly in temperate or cold climates, whereas hardwoods or angiosperms can be found in most regions of the world. A large number of hardwood species grow in the tropics (Dadswell and Hillis, 1962).

Hardwood xylem is often structurally complex (Esau, 1965). Its composition includes fibres for mechanical strength, thick-walled fibre-tracheids and large diameter vessels for water transport. Thin-walled tracheids and axial and ray parenchyma may also be present. Softwood xylem is constructed predominantly of tracheids, but each tracheid is in contact with one or more ray cells (Esau, 1965). Resin ducts surrounded by epithelial cells are often observed in gymnospermous woods (Dadswell and Hillis, 1962).

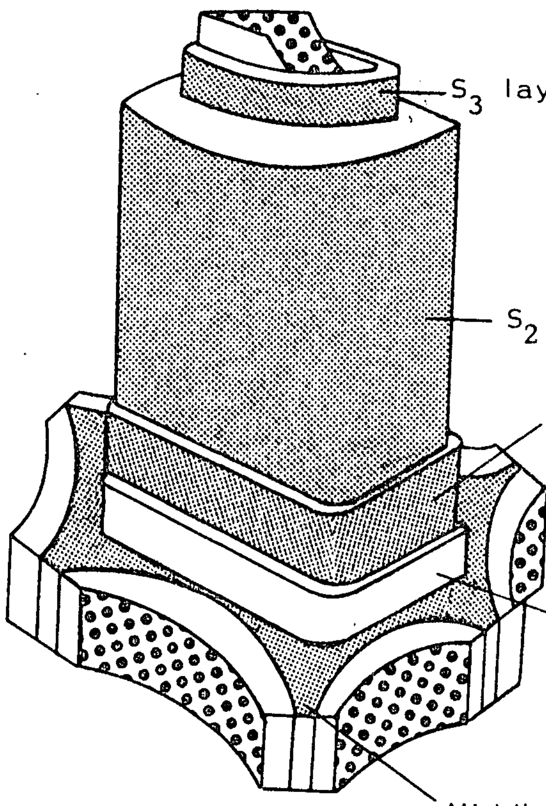
1.1.3 The Wood Cell Wall

Wardrop and Dadswell (1957), Wardrop (1964) and Coté (1977) have reviewed the structure and ultrastructure of the 'model' wood cell wall. The wall contains a middle lamella or intercellular region, a primary wall formed before cell radial expansion and elongation has ceased, and an inner secondary wall formed after cell expansion (Bailey and Kerr, 1935). The secondary wall may in turn be constructed of three identifiable layers, the S_1, S_2 and S_3; each with varying orientations of the cellulose microfibrils (see Fig. one; Bailey and Kerr, 1935; Wardrop and Dadswell, 1950). There may also be a tertiary layer which may or may not

Figure 1

Diagram showing the ultrastructure of a 'model' wood cell.

The S_1, S_2 and S_3 layers comprise the secondary cell wall.



from Coté (1977)

include the S_3 layer of the secondary wall (Wardrop and Dadswell, 1957; Liese, 1960, 1977).

1.1.4 Chemical Distribution of Cell Wall Constituents

The chemistry of wood cell walls has been reviewed by Meier (1964). He reported that the cellulose content for birch, pine and spruce woods was lower in the middle lamella and primary wall region (compound middle lamella) (Lange, 1950; Preston, 1952; Frey-Wyssling, 1964; Meier, 1964; Berlyn and Mark, 1965). As a result of the large volume of the secondary cell wall, it may contain 70-80% of the total wall lignin in birch vessels and fibres (Fergus and Goring, 1970). Coté (1977) showed that the highest concentration of cellulose was in the S_2 layer of the secondary wall of Scot's pine tracheids.

The lignin concentration of several woods was reported to be higher in the S_1 than the S_2 region (Bentum, Coté, Day and Timell, 1969). The S_3 layer of wood cell walls is considered somewhat resistant to microbial attack due to lignin type and concentration (Nilsson, 1976; Liese, 1977).

1.1.5 The Eucalypts

Boas (1947) and Penfold and Willis (1961) give detailed descriptions of the Eucalyptus hardwoods used for commercial timber production in Australia. The following description is quoted from Dadswell (1972):-

> "The genus Eucalyptus contains about 500 species ranging from dwarf mallees to very large trees, and inhabiting a great range of ecological sites. The genus with one exception (E. deglupta) is confined to Australia. Many species produce timbers of commercial importance.

The sapwood in all species is comparatively narrow - generally 2.5cm or less in width and pale in colour. The heart-wood colour varies from pale-brown to chocolate brown, although it may be straw-coloured in a few instances. The wood is quite dense (range from 0.48-0.93g/cm^3). The texture is moderately coarse whilst the grain is characteristically interlocked. 'Kino' veins (traumatic vertical canals) are a predominant feature of most of the genus.

The vessels in the majority of Eucalyptus species are solitary and variable in size (64-245 μm in different species). The vessel member length can range from 0.28-0.67mm. The number of vessels can vary from 5-11 per mm in transverse section.

Rays may number from 7-16 per mm in transverse section. Fibres are medium to thick-walled with member length varying from 0.75mm to 1.36mm and diameter 12 μm to 21 μm in different species. Xylem parenchyma may be either paratracheal (around vessels) or apotracheal (diffuse in wood)."

1.2 FUNGAL DECAY OF HARDWOODS

Historical aspects of wood decay have been reviewed by Cartwright and Findlay (1958), J. Levy (1965a) and Wilcox (1970). Buller (1909) and Hubert (1924) also wrote interesting accounts of the early investigative work.

The connection between wood decay and fungal growth was initially made by Theodore Hartig (1833). Subsequently, Robert Hartig (1878) detailed the relationship between basidiomycetous fungi and wood decay. He observed that an individual fungus could cause a characteristic decay pattern, and that the type of decay had a fairly constant appearance in different species of woods. Working with coniferous and hardwood timbers, he noted two fundamental types of attack: some fungi could remove cellulose and leave "wood-gum" (i.e. brown rots) whilst other fungi dissolved or delignified the wood (white-rotting organisms). Cartwright and Findlay (1943, 1958) detailed some of the subsequent developments in wood decay research and descriptions of the wood rots caused by basidiomycetous fungi are given by Cartwright and Findlay (1958) and Liese (1970).

The role of the non-Basidiomycete fungi in wood decay has been reviewed by Cartwright and Findlay (1958), J. Levy (1965) and C.R. Levy (1978). Schacht (1863) observed fungal hyphae enclosed in cell-wall cavities with pointed ends. Later, Bailey (1913) noticed fungi producing helically-orientated cavities within the latewood cells of Pinus taeda L. Bailey and Vestal (1937) gave the first detailed descriptions of conical-shaped cavities in wood. Tamblyn (1937) observed similar formation in cell walls of Jarrah (eucalypt) hardwood in Australia.

Findlay and Savory (1950) first described the soft-rot or superficial attack of wooden cooling-tower slats caused by the action of microfungi. Savory (1954a) isolated microfungi including Chaetomium globosum and Trichoderma viride from degraded cooling-tower slats. Cavities were observed within the secondary walls of woods examined.

Savory (1954b) believed that soft-rot attack of wood would only be of importance in situations where suitable nutrients were available or where physical conditions were unsuitable for the growth of the generally more aggressive wood-degrading Basidiomycetes. Oxygen supply (Savory, 1954a) and high surface area to volume ratios of timbers (e.g. boat hulls, Becker and Kohlmeyer, 1958) were thought to be factors aiding soft-rot attack. Savory (1954b) considered microfungi to be more tolerant to preservatives than Basidiomycetes; that is the preservative treatments adequate to prevent Basidiomycete attack might be insufficient to protect wood from soft-rot degradation. J. Levy (1965a) noted that soft-rot appeared to occur in all old timber exposed to moist conditions.

Soft-rot degradation has been observed in marine-situated woods (Barghoon and Linder, 1944; Johnson, 1956; Jones, 1962; Leightley, 1980a). Soft-rot is more commonly found in timber cut from hardwoods than from coniferous woods (Meier, 1955; J. Levy, 1965a; Nilsson, 1973), but all timbers are susceptible to attack. Butcher (1978) considered the soft-rot phase to be rare in softwoods due to Basidiomycetes rapidly replacing them in the fungal succession.

Soft-rot is often difficult to detect early in the infection stage (C. Levy, 1978). Surface softening may be detected using a sharp implement to dislodge chips of wood, showing distinct brash or carrotty fractures (Greaves, 1977; C. Levy, 1978). Deep soft-rot in Australian hardwood power-transmission poles is characterized by small pockets of decay some distance in advance of the main zone (Greaves, 1977).

1.2.1 Micromorphology of Soft-Rot Attack

Two basic forms of cell wall degradation caused by microfungi have been recognized (Corbett, 1965; Nilsson, 1973). Corbett described "Type one" attack as the formation of conical-shaped cavities moving helically around the S_2 layer of the secondary cell wall. "Type two" attack described erosion of the cell wall by fungal hyphae situated in the cell lumen (Corbett, 1965; Nilsson, 1973). This type of attack is found predominantly in hardwoods (Liese, 1970; Wilcox, 1970) as both the S_2 and S_3 layers of the secondary wall may be involved in hardwood decomposition (Courtois, 1963; Corbett, 1965). Some soft-rot fungi can produce both "Type one" and "Type two" attack (Nilsson, 1973).

Courtois (1963) described 14 different groups of fungal infection patterns recognizable on the basis of shape, size, position and orientation of the decomposed regions. The nature of the cell wall was considered to be the major factor distinguishing the cavity shape (Courtois, 1963; Willeitner, 1965). Liese (1970) regarded cavity shape and size to be determined by several factors including duration of fungal attack, fungal species, wood species, cell type and wood moisture content. He noted that the entire secondary wall can be interlaced with confluent cavities, leaving only the resistant middle lamella and tertiary wall.

Greaves (1977a) could not associate either wood structure or fungal species with different forms of attack. He noted that most studies on the morphology and histology of wood decay have described pure culture systems or alternatively, rigidly controlled conditions. Material obtained from the field is subject to groups of microorganisms acting in unison.

Soft-rot degradation is microscopically different from either brown or white-rot Basidiomycete attack. Wood attacked by brown-rotting organisms generally has the cell form or shape retained by the lignin framework as the cellulose is depleted (Cartwright and Findlay, 1943). Degradation is effected by enzymes diffusing through the tertiary wall (Liese, 1970). In comparison, progressive cell wall thinning due to loss of both cellulose and lignin is observed in white-rots (Wilcox, 1970).

1.2.2 Penetration of Wood Cell Walls by Soft-Rot Fungi

The initial means of fungal penetration into timber is generally through the ray cells or vessels (Corbett and Levy, 1963; Greaves and Levy, 1965). Two types of fungal penetration can be described: passive penetration (non-degradative penetration of the wood) and decay penetration (penetration and decay of the cell wall). Corbett (1963) considered passive penetration to proceed more easily from ray to tracheid than from tracheid to tracheid. The ray cell contents may be used as a nutrient source prior to decay penetration (Greaves and Levy, 1965).

The method of cell wall penetration by soft-rot fungi has been outlined by Corbett (1963, 1965) Levi and Preston (1965), J. Levy(1965a), Liese (1970) and Wilcox (1970). Fungal hyphae in the cell lumina produce a fine lateral branch or penetration hypha (Corbett and Levy, 1963) to grow through the tertiary layer of the cell wall. Hyphae may penetrate at a particular point due to a stimulus originating from the cell wall (Levy,1965a; Lündstrom, 1972). Penetration is accomplished either by enzymatic action (Proctor, 1941; Unligal and Chafe, 1974), or by mechanical pressure, or by a mixture of both methods (Cartwright and Findlay, 1958; Leightley and Armstrong, 1980).

Soft-rot hyphae can penetrate completely through the cell wall into the lumen of the adjoining cell <u>or</u> can form a T-branch ("axial" branch in the S_2 layer of the secondary cell wall) (Corbett and Levy, 1963; Levi and Preston, 1965). T-branching enables the hyphae to run parallel to the fibre axis (Liese, 1970) or to the cellulose micellae (Casagrande and Ouellette, 1971) or to the cellulose microfibrils in the cell wall (Bailey and Vestal, 1937; Lündstrom, 1974; Liese, 1970).

After T-branching, the hyphae follow the spiral fibrillar orientation (Liese, 1970) and produce enzymes causing cavity formation ("Type one" attack). Further cavities may be initiated by lateral hyphal branches from mature cavities or by fine hyphae from the tips of existing cavities (Corbett, 1963).

Bailey and Vestal (1937) and Meier (1955) noted the constant angularity of the conical cavity ends with the microfibril axis in a variety of timbers. Two theories have been proposed to account for the cavity shape:

(i) The structure of cellulose dictates cavity shape (Frey-Wyssling, 1938, 1956; Jutte and Wardrop, 1970). The conical shape may be dependent on various planes of hydrolysis along the cellulose microfibril.

(ii) Faster rate of diffusion of cellulose-degrading enzymes (cellulases) along the microfibril (longitudinal) axis as opposed to transverse diffusion (Roelofsen, 1956; Preston, 1979). Lignin deposition between the microfibrils was thought to have a retarding effect on the cellulases (Levi and Preston, 1965).

Soft-rot-like cavities have been found in a range of woody and non-woody fibres: cotton (Nilsson, 1974c), ramie fibres (Corbett, 1967), spruce holocellulose, Avicel cellulose, leaf and plant hair fibres and regenerated viscose fibres (Nilsson, 1974a), spruce bark (Parameswaran and Wilhelm, 1979), plant hairs and horse faeces (compost) (Baker, 1939), standing trees (Foster and Marks, 1968), birch, pine and spruce pulpwood (Henningsson, 1962).

Nilsson (1974c) stated, however, that soft-rot cavities were much more readily formed in lignified rather than lignin-free material.

Many theories have been advanced to explain the formation of conical cavity tips leading to intermittent cavity production. The narrowing between each cavity in a series is still produced even though the fungal hyphae run continuously (Wilcox, 1970). Corbett (1965) considered that hyphal elongation would slow whilst enlargement of the cavity behind the tip occurred. This view has been supported by Crossley and Levy (1977), Leightley and Eaton (1977) and Zainal (1978). Another explanation is that enzyme production might be alternately involved in the process of hyphal elongation and cavity enlargement (Levi and Preston, 1965). Other explanations include the restricted release of cellulolytic enzymes along certain sections of the hyphae (Courtois, 1963; Levi, 1965; Levi and Preston, 1965), catabolic repression of enzyme synthesis by cellobiose (Nilsson, 1974b) and enzyme production when hyphal alignment is parallel to the microfibrils (Corbett, 1965).

Type two soft-rot attack is similar to some Basidiomycete degradations (Levy, 1978). Cell wall erosion occurs predominantly in hardwoods (Levy and Stevens, 1966; Wilcox,

1970) as softwoods generally have a resistant S_3 layer which precludes this method of degradation (Nilsson, 1976). Enzymes are produced by fungal hyphae situated in the cell lumen. These enzymes act directly on the cell wall (Liese, 1970; Nilsson, 1976) and often produce characteristic V-shaped notches at branches from the longitudinal hyphae (Corbett, 1965; Levy, 1965b; Levy and Stevens, 1966; Wilcox, 1970). This erosion can often penetrate to the S_1 layer of the secondary cell wall or to the compound middle lamella (Corbett, 1965; Levy and Stevens, 1966).

Nilsson (1973) and C. Levy (1978) regarded cavity formation (Type one attack) to be restricted to a specialized group of microfungi, whereas the erosion form of attack appeared to be more common among imperfect fungi. Delignification of woods tended to encourage the erosion form of attack (Nilsson, 1974c).

1.2.3 The Soft-Rot Fungi

Savory (1954a) isolated 28 fungal species from various soft-rotted timbers; included were *Chaetomium globosum*, *Trichoderma viride*, and a *Phialophora* species regarded as a sapstain organism. Corbett (1963) listed the fungi thought to have the ability to induce soft-rot in hardwoods or softwoods in pure culture. Included in the list of eight Ascomycetes and 37 Fungi Imperfecti were *Cephalosporium* sp., *Phialophora richardsiae* and *Phialophora fastigiata* (Duncan, 1960).

Greaves and Savory (1965) reported the isolation of 184 identified fungal species from soft-rotted wood. A further

57 isolates were not identified. Duncan and Eslyn (1966) listed 66 species of Ascomycetes and Fungi Imperfecti belonging to 12 families. These isolates were capable of causing wood decay in pure culture conditions. Nilsson (1973) compiled a list containing 164 species of microfungi isolated from various degraded woods in Sweden. Gersonde and Kerner-Gang (1976) isolated 50 species of predominantly imperfect fungi from pine stakes.

Seehann, Liese and Kess (1975) published a list naming 305 true soft-rot fungal species; more than 100 were isolated from preservative-treated wood in ground contact. Henningsson and Nilsson (1976) identified 42 species of Ascomycetes and Fungi Imperfecti, 36 of which could produce cavities or erosion-type degradation in birch wood.

In Australasia, Greaves (1972a) isolated 52 fungal species from <u>Pinus radiata</u> and <u>Eucalyptus regnans</u> ground stakes; 12 of these species had confirmed decay capacity. Knox (1977) identified 35 species of soft-rot fungi isolated from New Zealand soils. All isolates caused measurable weight loss of silver birchwood. Line and Cruickshank (1979) isolated 42 species of microfungi in a survey of copper-chrome-arsenic (CCA)-treated hardwood transmission poles in Tasmania. Eight of these isolates could produce cavities in <u>Eucalyptus obliqua</u>. Leightley (1980b) isolated 27 species of Fungi Imperfecti and Ascomycetes from CCA-treated <u>Eucalyptus</u> sp. poles in Queensland. Eight of these species had the capacity to produce cavities in <u>Pinus elliottii</u> and <u>Eucalyptus maculata</u>.

Species of the Hyphomycete Phialophora have been implicated in the soft-rot decay of treated timber in Sweden (Nilsson and Henningsson, 1978) and Phialophora mutabilis has been found to be predominant amongst the destructive organisms in treated transmission poles in Australia (Line and Cruickshank, 1979; Leightley and Armstrong, 1980).

1.2.4 The Sap-Stain Fungi

Members of the Ascomycetes and Fungi Imperfecti can cause discoloration of woods (Hedgcock, 1906; Hubert, 1924; Verrall, 1939; Keirle, 1980) without large decreases in timber strength (Cartwright and Findlay, 1958; Liese, 1970). Some of these fungi may produce soft-rot decay under certain environmental conditions (Duncan, 1960; Krapivina, 1960; Wilcox, 1970). Further, some mould fungi that have little effect on the cell walls of one wood species, can produce soft-rot in another species (Merrill, 1965).

The main form of deterioration is wood discoloration caused by pigment within the penetrating hyphae or surface-formed conidia (Butcher, 1968a; Wilcox, 1970). The fungi use the wood substrate primarily as a habitat and draw most of their nutrient requirements from stored materials (e.g. starches) in the wood (Savory, 1954b). The pits are used as paths to traverse cell walls (Ward, 1898) or bore-holes are made through the walls (Cartwright and Findlay, 1943; Savory, 1954b).

1.2.5 Cellulolytic and Non-Cellulolytic Soft-Rot Fungi

Nilsson (1973) observed that, of the fungal species tested, those producing an erosion-form of attack (Corbett's

Type two) on birch cell walls were also able to degrade Walseth or acid-swollen cellulose. About one-third of the fungi that produced cavities (Type one) could not degrade Walseth cellulose (Nilsson, 1974b). He termed these organisms "non-cellulolytic" soft-rot fungi. The inability of the organisms to clear swollen cellulose was not attributable to weak cellulolytic activity, temperature factors, lack of growth factors, requirement of start-glucose or cell-bound enzymes.

The possible explanations he considered were: problems in cellulase induction, regulation of cellulase production by wood structure, and unsuitable cultural conditions.

1.3 BACTERIAL DEGRADATION OF WOOD

Literature on bacterial wood degradation has previously been reviewed by Rossell, Abbot and Levy (1973). Bacterial attack on cellulose was demonstrated by van Iterson (1904), whilst Rege (1927) noted that autochthonous soil bacteria could degrade rice-straw cellulose. Bacterial degradation and fermentation of birch, pine and aspen wood dusts was reported by Virtanen (1946), although the author believed that the grinding of the woods may have enhanced decomposition by removing some of the protective lignin barrier around the cellulose fibrils. Later, however, Hajny, Gardner and Ritter (1951) successfully fermented unground sawdust.

Increased permeability of water-stored woods due to bacterial action has been reported by Ellwood and Ecklund (1959), Knuth and McCoy (1962), Groot and Sachs (1976) and

Sharma and Kumar (1979). Knuth (1964) isolated Aerobacter spp. (now Enterobacter spp.), Bacillus polymyxa, B. subtilis and Pseudomonas spp. from a variety of woods. None of the isolates appeared to affect the tensile strength of the hardwoods and softwoods tested, but bacterial colonies were observed in the pit area. Wood permeability may be increased as a result of bacterial action on the pit tori (Knuth, 1964; Liese and Karnop 1968; Johnson, 1979; Groot and Sachs, 1976; Schink, Ward and Zeikus, 1981).

Both Verrall (1969) and Greaves (1970) believed that bacterial colonization generally occurred on the starch-rich parenchymatous ray cells. However, Harmsen and Nissen (1965) and Boutelje and Kiessling (1964) noted bacterial-caused structural deterioration of oak, pine and fir woods.

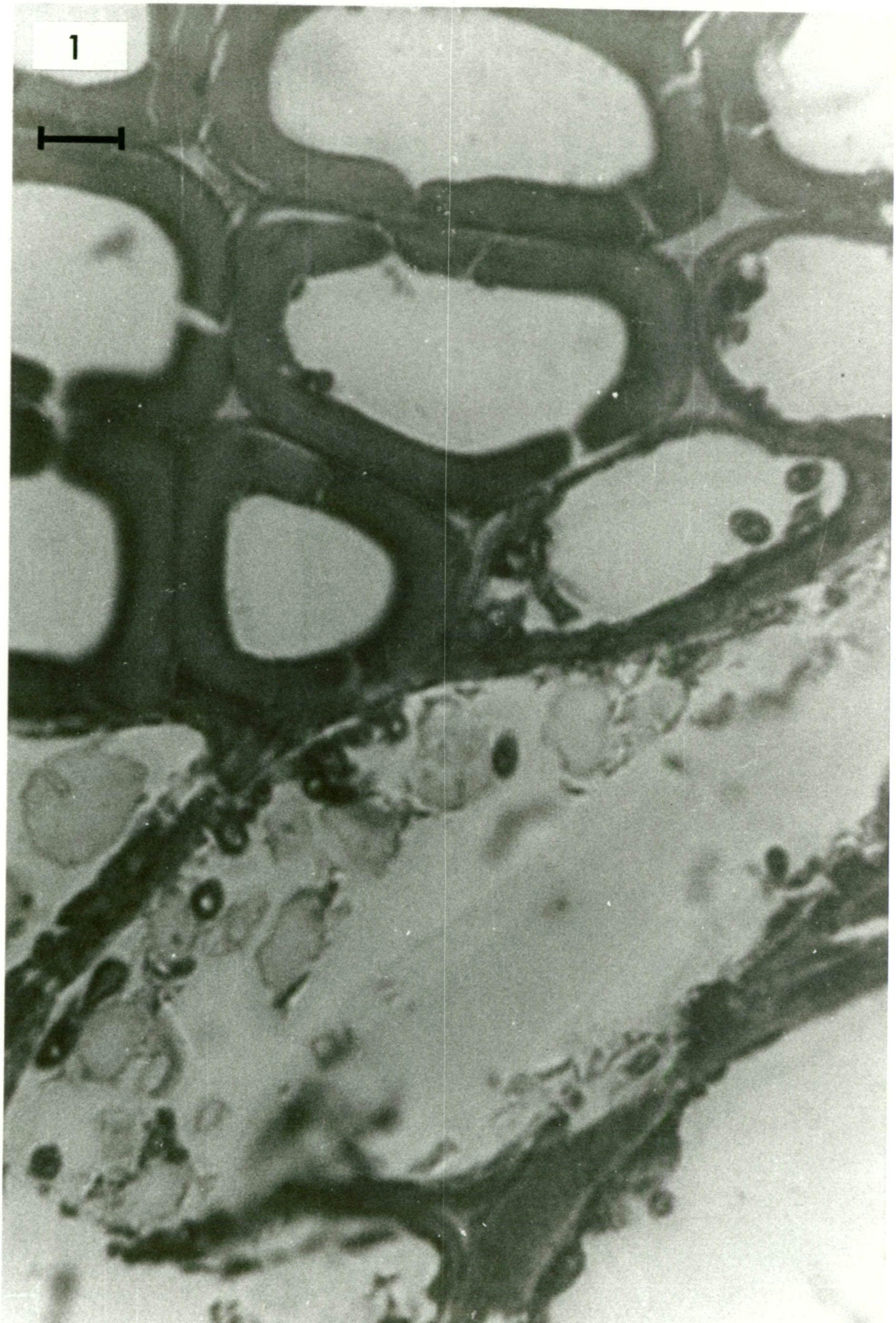
The lignin content of woods is inhibitory to bacterial attack of cell walls (Liese and Karnop, 1968; Holt and Gareth-Jones, 1978; Schmidt, 1980). Pure culture studies have demonstrated some attack on lignin (Trojanowski, Haider and Sundman, 1977; Kaplan and Hartenstein, 1980; Crawford and Crawford, 1980). Further, some Actinomycetes may produce significant weight losses in wood (Butcher, 1968a; Greaves, 1970; King, Eaton and Baecker, 1978; Baecker and King, 1980).

Bacterial attack of wood will occur in saturated or wet situations: log ponds, rivers, soil, marine sites, mines, cooling towers, etc. (Greaves, 1968; Rossell, Abbot and Levy, 1973). Bacterial colonization of ray parenchyma cells is common, however. If structural degradation of other cell types occurs, then bacteria action on the ray cells is usually well advanced (Wilcox, 1970).

Plate 1

A thin-section of soft-rotted CCA-treated *Eucalyptus* sp. transmission pole wood at the ground-line. Note the erosion of the parenchyma and two fibre cell walls, and the presence of bacteria in some cells.

Bar = 2.5μm.



1.4 PRODUCTION OF WOOD-DEGRADING ENZYMES BY MICRO-ORGANISMS

1.4.1 Cellulases

Linko (1977) noted the evolution of cellulose as a structural material for plant strength and rigidity, not as a carbohydrate reserve. The fibrillar structure of cellulose may hinder the access of large cellulase molecules (Cowling and Brown, 1969; Sihtola and Neimo, 1975; Fan, Lee and Beardmore, 1981). In addition, there appears to be a relationship between the degree of crystallinity of cellulose and resistance to degradation by microbial cellulases. Amorphous forms are highly susceptible to enzymatic hydrolysis (Walseth, 1952; Sasaki, Tanaka, Nanbu, Sato and Kainuma, 1979; Kanda, Wakabayashi and Nisizawa, 1980). Such amorphous regions lacking cross-linkages could exist along cellulose chains, allowing ready access to enzyme action (Hofsten, 1975; Sihtola and Neimo, 1975). "Swelling" or hydration of the cellulose fibril may also be necessary for enzymic activity (Sihtola and Neimo, 1975; Paquot, Thonart, Jacquemin and Rassel, 1981).

1.4.1.1 The mechanism of enzymic attack on cellulose

The cellulolytic activity of many organisms has been studied intensively. Included are the

(i) imperfect fungi: Trichoderma viride (Berghem and Pettersson, 1973; Petterson, 1975; Berg and Petterson, 1977); Trichoderma koningii (Wood and McCrae, 1972, 1975, 1978; Halliwell, 1975); Sporotrichum pulverulentum (formerly Chrysosporium lignorum) (Eriksson and Petterson, 1972;

Eriksson, 1978); *Fusarium solani* (Wood and McCrae, 1977); *Phialophora malorum* (Berg, 1978); *Aspergillus niger* (Hurst, Sullivan and Shepherd, 1977); *Aspergillus fumigatus* (Stewart and Parry, 1981);

(ii) Ascomycete fungi: *Thermoascus aurantiacus* (Tong, Cole and Shepherd, 1980); *Chaetomium thermophile* var. *dissitum* (Eriksen and Goksøyr, 1976);

(iii) bacteria: *Acetivibrio cellulolyticus* (Khan, 1980; Saddler and Khan, 1980); *Cytophaga* sp. (Chang and Thayer, 1977); *Cellvibrio fulvus* (Berg, 1975); *Pseudomonas fluorescens* var. *cellulosa* (Yamana, Suzuki and Nisizawa, 1970).

Reese, Siu and Levinson (1950) suggested a two-stage mechanism of cellulose hydrolysis:

$$\underbrace{\text{Native cellulose} \xrightarrow{C_1} \underbrace{\begin{array}{c}\text{shorter, linear}\\ \text{polyanhydro-}\\ \text{glucose chains}\end{array} \xrightarrow{Cx} \begin{array}{c}\text{soluble, small}\\ \text{molecules}\\ \text{capable of}\\ \text{diffusing into cell}\end{array}}_{\text{non-cellulolytic organisms}}}_{\text{cellulolytic organisms}}$$

They postulated that a non-hydrolytic enzyme (C_1) altered the cellulose crystallinity, facilitating the subsequent production of small-chain polyglucosides from the linear chains by Cx-enzymes. In this model, β-glucosides would hydrolyse the small-chain polyglucosides to glucose. The distinction was made between cellulolytic and non-cellulolytic organisms: truly cellulolytic organisms must possess the so-called C_1 factor to be able to attack crystalline cellulose (Wood, 1969; Enari and Markkanen, 1977).

The original model of sequential attack of cellulose proposed by Reese _et al_. (1950) has been disputed by various workers. At least three groups of enzymes are now believed to be involved in cellulose decomposition: endo-β-1,4-glucanases (EC 3.2.1.4., Cx enzymes), exo-β-1,4-glucanases (EC 3.2.1.91., the C_1 factor or enzymes) and β-glucosidases (cellobiases, EC 3.2.1.21.) (Shewale and Sadana, 1978).

The presently accepted view is that the C_1 factor is an exoglucanase of the cellobiohydrolase type (Berghem and Pettersson, 1973; Pettersson, 1975; Enari and Markkanen, 1977; Wood and McCrae, 1977).

The endoglucanases act randomly along the cellulose chains producing cello-oligosaccharides which are the substrates for exo-type enzymes (Hofsten, 1975; Streamer, Eriksson and Pettersson, 1975; Eriksson, 1978).

Pettersson (1975) proposed the following plan describing the attack of cellulose by _Trichoderma viride_.

Native (crystalline cellulose) $\xrightarrow{\text{Endoglucanase}}$ altered cellulose* with free chain ends

cellulose* $\xrightarrow{\text{Exoglucanase}}$ cellobiose

cellobiose $\xrightarrow{\beta\text{-glucosidase}}$ 2-glucose

The endoglucanases attack regions of low crystallinity in the cellulose fibre, consequently creating free chain ends

(Eriksson, 1978). Exoglucanases (cellobiohydralases) commence the degradation from the chain ends by hydrolytically removing cellobiose units. Finally, cellobiose is hydrolysed to glucose by a β-glucosidase.

Variations in the molecular weight of the endoglucanases may help to explain their mode of action. The molecular weights of these enzymes may vary from 12,500 (Berghem, Pettersson and Axio-Fredricksson, 1976) to 20,000 (Hakansson, Fagerstam, Pettersson and Andersson, 1978) through to 78,000 (Tong, Cole and Shepherd, 1980). Thus the smaller molecular weight enzymes may, because of molecular size and hence greater accessibility, be able to initiate attack on crystalline cellulose.

The endoglucanases, exoglucanases and β-glucosidases of organisms can often show strong synergistic activity on crystalline cellulose, but little individual degradative action (Selby and Maitland, 1967; Streamer, Eriksson and Petterson, 1975; Wood and McCrae, 1975; McHale and Coughlan, 1980). In addition, it is possible that enzyme-enzyme complexes between cellobiohydrolases and endoglucanases are formed on the surface of cellulose chains (Wood and McCrae, 1978).

Two oxidative enzymes of importance in cellulose degradation have recently been discovered. Eriksson, Petterson and Westermark (1975) described the action of a cellobiose oxidase from the white-rot fungus Sporotrichum pulverulentum. This enzyme utilizes molecular oxygen to oxidize cellodextrins (cellobiose → cellohexose in size) to form aldonic acids (Ayers, Ayers and Eriksson, 1978). Its function may be to decrease the cellobiose concentration as it is an inhibitor of some cellulases (Ayers *et al.*, 1978).

A degradative system involving a cellobiose-quinone oxidoreductase in lignified substrates was outlined by Westermark and Eriksson (1974). The enzyme, which needs quinone as a substrate (Eriksson,Pettersson and Westermark, 1975), is involved in the simultaneous degradation of both cellulose and lignin. It may not be present, however, in fungi other than white-rotting organisms (Reese and Mandels, 1980; McHale and Coughlan, 1980).

1.4.1.2 Induction and repression of cellulases

Cellobiose is probably the inducing agent for cellulases under sub-optimal conditions. It is inhibitory in moderate concentrations, however (Mandels and Reese, 1960; Howell and Stuck, 1975; Berg and Pettersson, 1977). Interestingly, natural inhibitors of cellulase activity have been extracted from *Eucalyptus* woods (Mandels and Reese, 1965).

Sophorose, a β-1,2-disaccharide of glucose, is a potent inducing agent *in vitro* for cellulase production (Mandels, Parrish and Reese, 1962). This effect may be limited to specific microorganisms, e.g. *Trichoderma* spp. and *Pseudomonas* spp. (Sternberg, 1976). Inductive formation of cellulase by sophorose is strongly repressed by glucose, glycerol and other readily metabolizable compounds (Nisizawa, Suzuki, Nakayama and Nisizawa, 1971).

1.4.1.3 Cell-bound/extracellular cellulases

There is variability between organisms in the nature of their cellulases, whether cell-bound or extracellular.

When degrading solid substrates, cells either come into direct contact with the substrate (an obvious requirement for cell-bound enzymes) or secrete extracellular enzymes which diffuse into the substrate. Metabolizable products released are utilized by the organisms (Binder and Ghose, 1978). Cellulolytic organisms are most efficient when cellulases are cell-bound, as production of extracellular cellulases necessitates loss of protein to the environment (Enari and Markkanen, 1977). Further, cell-bound enzymes may be more effective in hydrolysing insoluble substrates due to alignment of enzymes (Hofsten, 1975).

The existence of enzymes in culture solutions does not necessarily mean that they are extracellular in nature; it may be the result of cell lysis (Pollock, 1962). For example, extracellular cellulases from Trichoderma viride have only been found in the stationary and post-stationary growth phases (Berg and Pettersson, 1977), suggesting cell lysis. Further, Deshpande, Eriksson and Pettersson (1978) noted that cellulose was needed in the culture medium for induction of extracellular cellulase by Sporotrichum pulverulentum.

Studies with bacteria indicate that both extracellular and cell-bound components of the cellulase system occur (Beguim, Eisen and Rompas, 1977; Thayer, 1978; Saddler and Khan, 1980). Berg (1975) observed that the cell-bound cellulase of Cellvibrio fulvus was only loosely adhering to the cell surface; hence he coined the term "surface-bound" enzyme.

1.4.1.4 Measurement of cellulase activity

The entire cellulase complex cannot be characterised by a single method (Leisola, Linko and Karvonen, 1975; Ng and Zeikus, 1980). However, cellulase activity may be assessed in various ways:

(a) by the use of soluble substrates, e.g. sodium carboxymethyl cellulose (NaCMC) (Reese, Siu and Levinson, 1950) or the non-ionic hydroxyethyl cellulose (Child, Eveleigh and Seiben, 1973). The activity may be measured by reduction in viscosity of the soluble substrate (Levinson and Reese, 1950; Sherwood and Kelman, 1964; Almin and Eriksson, 1967) or, alternatively, the resultant reducing sugars produced may be measured (Reese, Siu and Levinson, 1950; Miller, Blum, Glennon and Burton, 1960).

(b) by the clearing of acid-swollen cellulose (Walseth, 1952; Rautela and Cowling, 1966; Stranks and Bienada, 1971).

(c) by the attack on crystalline cellulose; including filter paper (Mandels and Weber, 1969; Griffin, 1973; Wabnegg, Messner and Röhr, 1980); cotton (Nilsson, 1974c; Line and Cruickshank, 1979) and microcrystalline cellulose, e.g. Avicel, Solka floc (Nilsson, 1974b).

(d) by the release of soluble coloured compounds from insoluble dyed substrates (Fernley, 1963), e.g. dyed microcrystalline cellulose (Avicel, Solka floc) (Leisola, Linko and Karvonen, 1975; Leisola and Linko, 1976); dyed chromatography paper (Poincelot and Day, 1972); dyed cellophane (Moore, Basset and Swift, 1979) and cellulose azure (Smith, 1977).

(e) by the use of various new techniques including gas-liquid chromatography of silylated reaction products (Storer, Gawthorne, Francis and Illman, 1979); polarographic assay involving oxygen consumption (Green, Han and Anderson, 1977); immunoelectrophoretic detection (Nummi, Niku-Paavola, Enari and Raunio, 1980), and radiometric microassay (Vardanis and Finkelman, 1981).

There is often little correlation between the methods used for measuring cellulase activity (Rautela and Cowling, 1966; Nilsson, 1974b; Canevascini and Gattlen, 1981). Measurement of CMC-ase activity is probably an estimate of Cx or endoglucanase activity (Reese, Siu and Levinson, 1950). Thus, the C_1 or exoglucanase activity is not estimated by this method (Wood, 1969; Nilsson, 1974b). Goksøyr (1975) did state, however, that the endoglucanases were the central cellulolytic enzymes in all known systems.

Nilsson (1973) regarded the ability of organisms to degrade acid-swollen or Walseth cellulose to be dependent on their capacity to produce exoglucanases (C_1 factor) and not endoglucanase alone. Tanaka, Taniguchi, Morita, Matsuno and Kamikubo (1979) investigated the effect of the crystallinity of acid-swollen cellulose on the degree of its solubilization by cellulases. They found that the degree of solubilization increased linearly with the decrease of crystallinity under the same treatment conditions.

The most difficult step in enzymic cellulose hydrolysis is solubilization or removal of cellulose crystallinity (Enari and Markannen, 1977). Dyed cellulose substrates have been employed to measure this solubilization (Leisola, Linko and

Karvonen, 1975). Substrates of reasonably high crystallinity, e.g. filter paper, or Avicel, should be used rather than Solka floc or cellulose azure for this purpose (Leisola, Linko and Karvonon, 1975). However, the presence of dye bonding to the glucose groups of the cellulose does cause a large degree of inhibition to hydrolysis (Leisola and Linko, 1976).

It would appear that if the total or overall cellulolytic activity of microorganisms is to be estimated, then the ability to attack crystalline cellulose (cotton, filter paper) should be investigated (Enari and Markkanen, 1977).

1.4.2 Hemicellulose-Degrading Enzymes (Hemicellulases)

Dekker and Richards (1976) gave a detailed review of the occurrence, purification, physico-chemical properties and modes of action of the hemicellulases. Earlier, Sørensen (1957) reviewed research on D-xylanases to that time.

Dekker and Richards (1976) defined the hemicellulases as enzymes that specifically degrade only the hemicelluloses. This does not include the glycosidases, although these may be capable of hydrolysing the short-chain appendages from the main backbone chain of hemicelluloses.

A wide range of organisms are reported to be capable of producing hemicellulases: fungi, bacteria, some invertebrates (e.g. *Helix* sp., snail), some plants (seeds and algae).

1.4.2.1 Xylanases

Xylanases are hydrolytic enzymes capable of hydrolysing (1→3)-β-D-xylans to D-xylose [exo-(1→3)-β-D-xylanases EC 3.2.1.72] and D-xylo-oligosaccharides [endo-(1→3)-β-D-xylanases EC 3.2.1.32] (Dekker and Richards, 1976). Many workers have investigated the mode of action of xylanases from various groups of organisms, including: *Polyporus schweinitzii*, *Chaetomium globosum*, *Aspergillus wentii* (Keilich, Bailey and Liese, 1970; *Fuasrium roseum* (Gascoigne and Gascoigne, 1960); *Trichoderma reesei* (Tangnu, Blanche and Wilke, 1981); *Streptomyces* sp. and *Micromonospora* sp. (Sørensen, 1953). All were reported to be either endo- or exo- in their mode of action.

A β-xylosidase (EC 3.2.1.37) was reported to be produced by *Aspergillus niger* (Takenishi, Tsujisaka and Fukumoto, 1973), whilst endoxylanases and xylosidases were reported from *Coniophora cerebella* (King and Fuller, 1968).

Fungal D-xylanases may be of the endo- or exo- form but bacterial xylanases are generally of the endo- type (Dekker and Richards, 1976).

Lyr (1959) considered D-xylanases to be produced by wood-rotting fungi on any carbon source; the enzymes were constitutive. This was supported by Bucht and Eriksson (1968) and Eriksson and Rzedowski (1969), although sophorose was later thought to have an inductive influence (Nisizawa, Suzuki and Nisizawa, 1971).

1.4.2.2 Mannanases

Mannanases are primarily of the endo-type (EC 3.2.1.78). These enzymes are capable of hydrolysing the 1→4-β-D-mannopyranosyl linkages of D-mannans and D-galacto-D-mannans (Dekker and Richards, 1976). Mannanase activity has been investigated in *Chaetomium globosum* and *Aspergillus* wentii (both endohydrolases) (Keilich, Bailey and Liese, 1970); Aspergillus fumigatus, Aspergillus giganteus, Cladosporium olivaceum, Paecilomyces varioti, Penicillium spp. (all endo-type enzymes) (Reese and Shibata, 1965).

The induction of cellulase, xylanase and mannanase may be controlled by a single regulator gene in some fungi (Eriksson and Goodell, 1974; Highley, 1976). Mannans have been reported to be better inducers of xylanase, carboxymethyl cellulase and mannanase than xylan (Keilich, Bailey and Liese, 1970; Highley, 1976).

1.4.2.3 Arabinases

Most L-arabinan-degrading enzymes are of the exo-type, although both endo- and exo-forms have been reported (Dekker and Richards, 1976).

1.4.2.4 The significance of hemicelluloses in wood decay

Lyr and Novak (1961) investigated the potential for cellulase, xylanase and mannanase production by strains of ten species of Fungi Imperfecti. All cellulolytic species also produced xylanase and mannanase. In a survey of more than 300 soil fungi, Domsch and Gams (1969) noted that xylan was more readily attacked than carboxymethyl cellulose. One species, Oidiodendron echinulatum specifically degraded xylan. The ability to attack xylan is widespread amongst wood-degrading bacteria (Deschamps and Lebeault, 1980).

Nilsson (1974b) regarded simultaneous production of hemicellulases with cellulases as likely, since production of one enzyme only by an organism would restrict the ability to degrade either hardwood or softwood substrates [xylan is the main hardwood hemicellulose, mannan is the principal softwood hemicellulose (Timell , 1964)].

In a study of 36 species of wood-attacking microfungi, Nilsson (1974b) assumed that the production of cellulases, xylanases and mannanases occurred by these organisms. Failure to detect one or more of the three cell-wall degrading enzymes was thought to be the result of deficiencies in the test method employed.

Differences in the susceptibility of hardwoods and softwoods to attack by soft-rot, brown-rot and white-rot fungi may be due to the composition of the hemicelluloses in the woods (Keilich, Bailey and Liese, 1970; Highley, 1976). Takahashi and Nishimoto (1973) related the production of xylanases (xylan as sole carbon source) by fungi, to the ready attack of hardwoods in comparison with softwoods. Preferential utilization of the main hemicellulose in softwoods (glucomannan) by brown-rot fungi and a similar relationship between hardwoods (xylan) and soft-rot fungi may be a factor in the predominant occurrence of brown-rotting Basidiomycetes and soft-rot fungi on softwoods and hardwoods respectively (Keilich, Bailey and Liese, 1970; Hulme and Butcher, 1977; Rypácek, 1977).

Hemicelluloses may form a sheath around the cellulose microfibrils in the secondary wall (Bauer, Talmadge, Keegstra and Albersheim, 1973). This possibility was supported by the work of Levy (1978) who obtained a marked relationship between pentosan (hemicellulose) content, and soft-rot decay rating of Papua-New Guinea timbers. Thus he reasoned that the high pentosan content of some woods may be a predisposing factor in soft-rot attack.

1.4.3 Lignin-Degrading Enzymes

Literature on the microbial attack of lignin has been reviewed by Kirk (1971), Higuchi (1971), Ander and Eriksson (1978), Crawford and Crawford (1980), Kaplan and Hartenstein (1980) and Kirk, Higuchi and Chang (1980).

White-rotting Basidiomycetes and some Ascomycetes degrade lignin and carbohydrate simultaneously, whilst brown-rot organisms cause extensive depletion of carbohydrate only (Kirk, 1971). The clear physiological distinction between the brown-rot and white-rot fungi appears to be the completeness of their lignin-degrading enzyme systems (Kirk, 1971). Lignin degradation by white-rot fungi proceeds by way of extracellular mixed function oxygenases and deoxygenases which mediate demethylation, hydroxylation and ring-fission reactions (Crawford and Crawford, 1980).

The essential phenol-oxidizing enzymes that are part of the lignin-degrading complement of white-rot fungi are: laccase (O_2 : P-diphenyl oxidoreductase EC1.10.3.2); peroxidase (O_2 : diphenol oxidoreductase EC 1.11.1.7); tyrosinase (O_2 : diphenol oxidoreductase EC 1.10.3.1) (Kirk 1971).

The brown-rot basidiomycetous fungi may alter the lignin structure, as a change in methoxyl content has been reported (Kirk, 1971). The Bavendamm (1928) test has been extensively used to characterize wood-rotting Basidiomycetes into white (lignin-degrading) or brown-rotting types. White-rot fungi, when cultivated on an agar medium containing gallic or tannic acid, produce a dark coloured zone around the mycelial mat, whereas brown-rot fungi do not form the coloured zone. Davidson, Campbell and Blaisdell (1938) examined 210 species of wood-rotting fungi; 96% were white-rotting organisms producing a positive Bavendamm test. The presence or absence of a laccase enzyme is now thought to be an important criterion in the classification of decay fungi (Higuchi, 1971).

The capacity of soft-rot fungi to degrade lignin has been reported to vary from substantial (Levi and Preston, 1965) to minimal (Seifert, 1966). Eslyn, Kirk and Effland (1975) examined six strains of imperfect fungi for lignolytic activity. They found that all species (including *Graphium* and *Paecilomyces* spp.) could attack and deplete the lignin, but rates of degradation varied.

Working with ^{14}C-labelled substrates, Haider and Trojanowski (1975) considered that soft-rot species tested (including *Chaetomium* and *Stachybotrys* spp.) could cause considerable degradation of lignin by attacking the methoxyl side-chain and aromatic groups of the polymer. No evidence of laccase activity was found and the peroxidase activity was lower in the soft-rot fungi than in some Basidiomycetes tested in parallel. Kuster and Little (1963) did, however, find laccase activity in the imperfect fungi *Aspergillus fumigatus* and *Penicillium spinulosum*. Later, Drew and Kadam (1978) discovered that *Aspergillus fumigatus* could metabolize 'Kraft' lignin. An isolate of *Fusarium solani* was able to attack ^{14}C-synthetic lignins and use them as sole carbon sources (Norris, 1980). However, the actual extent and pathways of lignin degradation by soft-rot fungi are largely unknown (Crawford and Crawford, 1980).

Early techniques used to investigate bacterial attack of wood lignins have been questioned (Kirk, 1971). There is evidence, nevertheless, that few bacterial species have the ability to attack intact lignin in wood (Greaves, 1971; Ander and Eriksson, 1978). Some prior delignification may be needed before bacterial strains can attack woods (Holt and Gareth Jones, 1978; Schmidt, 1978, 1980). Recently, however, workers have reported that species of *Nocardia* (Trojanowski, Haider and Sundman, 1977), *Pseudomonas* (Haider , Trojanowski and Sundman, 1978) and *Streptomyces* (Crawford, 1978; Phelan, Crawford and Pometto, 1979) can degrade ^{14}C-labelled synthetic lignins (dehydro-polymers of coniferyl alcohol [see Kirk, Connors, Bleam, Hackett and Zeikus, 1975]) *or* ^{14}C-labelled lignin in intact woods (Crawford and Crawford, 1976; Crawford, Crawford and Pometto, 1977). Demethoxylation of lignin structures is probably an important initial step in bacterial degradation (Crawford and Crawford, 1980).

1.4.4 Pectic Enzymes

Bateman and Miller (1966), Fogarty and Ward (1974), Rexová-Benková and Markovic (1976) and Romberts and Pilnik (1980) give detailed reviews of the mode of action, occurrence, purification and assay of pectic enzymes. These enzymes are present in a wide range of plants, fruits, stems, roots, leaves, fungi and bacteria (Bateman and Miller, 1966; Rexová-Benková and Markovic, 1976).

The substrates of pectic enzymes (pectin and pectic acid) are branched heteropolysaccharides; the backbone of which contain L-rhamnose residues and alpha-D-(1-4) linked residues of D-galactopyranosiduronic acid. The neutral sugars D-galactose, L-arabinose and occasionally D-xylose and L-fucose may form side-chains off the pectin molecule (Rexová-Benková and Markovic, 1976).

The pectin-esterases (EC 3.1.1.11) de-esterify the methyl esters of pectin. These enzymes proceed along the molecular chain producing blocks of free carboxyl groups (Rexová-Benková and Markovic, 1976).

The D-galacturonases (EC 3.2.1.15) catalyse the hydrolytic cleavage of the glycosidic alpha-D-(1-4) bonds of non-esterified D-galactopyransiduronic residues. This activity results in pronounced reduction of the viscosity of a substrate solution (Sherwood and Kelman, 1964; Sherwood, 1966; Rexová-Benková and Markovic, 1976). Both endo- and exo-enzyme forms have been characterised (Mill, 1966).

Pectin lyases (EC 4.2.2.2) catalyse the cleavage of alpha-D-(1-4) glycosidic bonds of galacturans by beta-eliminative degradation (Albersheim, Neukom and Deuel, 1960). Both endo- and exo-pectate lyases have been characterised (Rexová-Benková and Markovic, 1976).

Pectic enzymes appear to be inducible (Perley and Page, 1971). Galacturonic acid has been reported to induce endopolygalacturonase with <u>Verticillium albo-atrum</u> and <u>Fusarium oxysporum</u> f.sp. <u>lycopersici</u> (Cooper and Wood, 1973).

Fungal pectin enzymes characterised include those of the following fungi: <u>Aspergillus niger</u> (Mill, 1966; Sherwood, 1966); <u>Fusarium roseum</u> (Perley and Page, 1971); <u>Fusarium oxysporium</u> f.sp. <u>lycopersici</u> (Sherwood, 1966; Cooper and Wood, 1973); <u>Geotrichum candidum</u> (Barash and Eyal, 1970); <u>Verticillium albo-atrum</u> (Cooper and Wood, 1973); <u>Monilinia</u>

fructicola, *M. laxa*, *Sclerotinia sclerotiorum*, *S. minor*, *Botrytis cinerea*, *B. gladiolorum*, *B. fabae*, *B. allii*, *Penicillium atrovenetum* (Cruickshank and Wade, 1980).

Reports describing bacterial pectic enzymes have been made by Nagel and Vaughn (1962) [*Bacillus polymyxa*]; Karbassi and Luh (1979) [*Bacillus* sp.]; Okamoto, Hatanaka and Ozawa (1964) [*Erwinia aroidea*]; Nasuno and Starr (1966) [*Pseudomonas marginalis*].

1.4.5 Amylases (Starch-Degrading Enzymes)

Aspects of the purification, structure, mode of action and assay of starch-degrading enzymes have been reviewed by Bernfield (1951), Greenwood and Milne (1968), Takagi, Toda and Isemura (1971), and Fogerty and Kelly (1979).

Starch is an energy-reserve polysaccharide of heterogeneous fractions of approximately 27% amylose (a linear chain of α-1,4-linked glucose units) and 73% amylopectin (a short-chain of 1,4-glucose units) bonded by 1,6 linkages to form a branched molecule (Meyer and Gibbons, 1951; Akazawa, 1965; Stecher, 1968). Amylases are widely found in mammals, higher plants, fungi, bacteria and crustaceans (Greenwood and Milne, 1968).

Three major amylase types have been well-characterized: α-amylases, β-amylases and glucoamylases. The prefixes α and β refer to product stereochemistry, not substrate specificity (Thoma, Spradlin and Dygert, 1971 ; Bilderback, 1973).

The α-amylases (α-1,4-glucan 4-glucanohydrolases, EC 3.2.1.1.) catalyse the random (endo-type) hydrolysis of α-1→4 glucosidic linkages of polysaccharides including starch glycogen (and degradation products) (Greenwood and Milne, 1968; Thoma, Spradlin and Dygert, 1971).

β-amylases (α-1→4-glucanmaltohydrolases, EC 3.2.1.2.) are exo-enzymes which break alternate glucosidic bonds, forming maltose (Thoma, Spradlin and Dygert, 1971). It is thought that β-amylases are unable to pass the branched 1,6 linkage of amylopectin (Akazawa, 1965). The third major class of amylases are the glucoamylases, exo-type enzymes that convert starch directly to D-glucose (Greenwood and Milne, 1968; Thoma, Spradlin and Dygert, 1971).

Strains of <u>Aspergillus</u> <u>oryzae</u> and <u>Bacillus</u> <u>polymyxa</u> are potent producers of amylases (Takagi, Toda and Isemura, 1971).

1.5 WOOD PRESERVATION

Historical aspects of wood preservation have been reviewed by Cartwright and Findlay (1958) and Purslow (1979). Early references on wood protective chemicals have been reported to date from 200BC (Greaves, 1980). General aspects of wood preservation are covered in reports by Findlay (1962), Richardson (1978) and Wilkinson (1979).

Wood preservatives should ideally possess the following characteristics: high toxicity to wood-destroying organisms, low volatility, high resistance to leaching and ability to penetrate deeply into wood. They should also be non-corrosive to metals and non-injurious to wood itself, safe to handle and economically viable (Cartwright and Findlay, 1958; Wilkinson, 1979).

(a') diffusion processes. At the time of treatment, the wood must have a high moisture content, preferably in excess of 50% of its dry weight. A high concentration of a water soluble or water miscible material is applied to the surface and allowed to diffuse into the wet wood.

Wood preservatives can be generally classified into three groups (Hof, 1981).

(a) preservative oils derived wholly or in part from coal tar or wood tar, e.g. creosote;

(b) water-borne chemicals, e.g. mercuric chloride, zinc chloride, copper sulphate, copper-chrome-arsenic, boric acid, fluoroborates;

(c) organic-solvent-borne preservatives, e.g. copper napthenate, tri-butyl tin oxide, pentachlorophenol.

1.5.1 Methods of Wood Preservative Application

Methods of application have been adequately reviewed by Cartwright and Findlay (1958) and Findlay (1962). In summary, some techniques in current use are: (See opposite)

(a) brushing, spraying, deluging or immersion (Findlay, 1962; Wilkinson, 1979);

(b) open tank or 'hot and cold' process (Findlay, 1962). Seasoned or green timber is submerged in a preservative bath to which heat is applied. Air is expelled from the wood and is replaced by preservative on subsequent cooling;

(c) pressure processes -

(i) full-cell or Bethell process (patented 1838). A period of vacuum is applied prior to impregnation with preservative (Cartwright and Findlay, 1958).

(ii) empty-cell processes in which <u>no</u> initial vacuum is drawn (Wilkinson, 1979). The Rueping process involves a period of air pressure prior to preservative flooding, whilst the Lowry technique has no such period. Both these latter methods were designed to minimize bleeding of liquids; a special problem with coal-tar preservatives.

As a result, the empty-cell treatment processes can be more economical than full-cell methods (Wilkinson, 1979). Variations on these pressure processes described are the oscillating and alternating pressure methods, the ultra-high pressure method, the double vacuum process and various solvent recovery processes (Wilkinson, 1979).

The following factors all influence the life of preserved timber: environment or conditions under which the timber is used, the inherent properties of the timber, i.e. its natural durability and treatability (see also Hulme and Butcher, 1977; Greaves, 1979a), the loading or retention of preservative in the wood, the macrodistribution of the wood preservative, i.e. depth of penetration and evenness of distribution through the zone of penetration, the micro-distribution of the preservative, i.e. deposition within cell wall as well as cell lumen, the chemical affinity between preservative and timber, i.e. whether it becomes attached at the microcellular level or whether it attacks or reacts with the wood in some way, the inherent properties of the wood preservative (chemical stability, toxicity, leachability, volatility and resistance to attack by microorganisms) (Cockroft, 1974).

1.5.2 Failure of Copper-Chrome-Arsenic (CCA)-Treated Hardwoods in Ground Contact

CCA-preservatives have been successful in controlling soft-rot attack in softwood timbers, but often exhibit poor control in the more susceptible hardwoods (Butcher, 1978; C. Levy, 1978). To date, three theories have been proposed to account for this anomaly:

(a) variability in hardwood anatomy compared with the more uniform softwood structure. Related to this is the treatability of hardwoods with preservatives (Greaves, 1972b,1974; Liese and Peters, 1977; Butcher, 1979a).

(b) Inadequate penetration of wood cell walls by fungitoxic preservative elements (Dickinson, 1974; Greaves, 1977).

(c) High substrate susceptibility of treated hardwoods to fungal attack (Hulme and Butcher, 1977; C. Levy, 1978; Butcher, 1980a). Nutrient substances may be more readily utilized by the degrading fungi in hardwoods than softwoods (C. Levy, 1978). Thus, CCA-treated hardwoods may be failing through soft-rot attack because of treatment to sub-toxic thresholds (Hulme and Butcher, 1977).

Other factors involved in attack of CCA-treated timber are fungal tolerance to CCA-type preservatives (Ross, 1975; Stranks and Hulme, 1976; Nilsson and Henningsson, 1978; Schmidt and Zeimer, 1977) and leaching of CCA-preservatives from woods in soil content (Leightley, 1980b).

1.5.4 Some Reports of New Preservatives and Preservation Methods

Potential new wood preservative agents include: a range of agricultural and industrial fungicides, including Skane M8 and Busan 52 (Hedley, Preston, Cross and Butcher, 1979); alkyl ammonium compounds (Butcher and Drysdale, 1978; Butcher, 1979b; Butcher, 1980b; Ruddick, 1981); tributyl tin compounds (re-investigated by Crowe, Hill, Smith and Cox, 1979) and silicon tetrachloride (Owens, Shortle and

Shigo, 1980). Also, the chemical modification of wood itself has shown promise in inhibiting fungal attack (Rowell, 1975; Lutomski, 1975; Peterson and Thomas, 1978; Stevens, Schalck and van Raemdonk, 1979).

1.5.5 Remedial Wood Preservation Treatments for Eucalypt Poles

Until effective preservatives for the protection of hardwoods in ground contact are developed, remedial treatments of transmission poles _in service_ must be considered (Greaves, 1977b).Greaves (1979b) and Line (1979) list some of the potential ground-line maintenance or remedial treatments for eucalypt poles in Australia:

(a) surface application of preservatives (usually creosote). In addition, soil surrounding poles may be 'sterilized' by 'puddling' with creosote or other preservatives (Keating, 1958; Beesley, 1963).

(b) use of the "Cobra" method for injection of preservatives into sapwood (Findlay, 1962; Chambers, 1963).

(c) 'dressing' (removal of infected wood) followed by surface application of preservative.

(d) sterilization _in situ_. Fumigation, steaming, irradiation or oxy-charring of poles (Cummins, 1935; De Campo, 1963).

(e) butt encapsulation to prevent colonization of the wood by soft-rot or other fungi (Johnstone,Gardner and Pitt , 1979).

(f) toxicant-containing cartridges bored into the wood (Bechgaard, Borup, Jermer and Henningsson, 1979).

(g) toxicant-containing bandages (Greaves, 1977a, 1979b, 1980; Dale, Greaves and Thornton, 1978).

(h) biological control (Ricard, 1975; Morris and Dickinson, 1981).

1.5.6 Methods for Assessing the Degree of Wood Degradation and the Effectiveness of Wood Preservatives in the Field

Historically, methods used to monitor wood degradation in the field include subjective visual assessment, sampling and subsequent microscopic analysis in the laboratory, and the subjective impaction assessment using a knife, screw-driver or hammer.

A non-destructive method involving measurement of the electrical conductivity of woods was outlined by Shigo and Shigo (1974). The Shigometer (R) device measured abrupt decreases in wood resistance to a pulsed electric current along a pre-drilled core path (Shigo and Chase, 1972; Shigo, Shortle and Ochrymowych, 1977). In laboratory tests, Thornton (1979) noted that the instrument was able to detect levels of Basidiomycete decay as low as 1.0% of the cell wall material. Leightley (1981) considered that the device gave little added information to visual assessment when inspecting soft-rotted or surface-degraded timbers, but was of considerable use for the detection of heart-rots (Basidiomycete degradation) in transmission poles.

Shigo (1980) mentioned two points to be noted when using the technique in the field: the timbers under test must be above fibre saturation point, and secondly, special care and proper operator interpretation is needed when using the device.

The Pilodyne (R), an instrument for non-destructive testing of the shock resistance of wood, has been developed in Scandinavia (Hoffmeyer, 1975, 1978). The instrument quantifies the penetration produced by a constant amount of energy delivered to a blunt pin by a coiled spring. It is ideally suited to studies on soft-rot attack and has advantages of speed of assessment and elimination of the need for skilled labour. One disadvantage is that readings obtained are dependent on moisture contents of the woods tested. There is no linear relationship between moisture content and penetration depth (Hoffmeyer, 1978).

The Pilodyne (R) has been accepted for use in Sweden and Denmark as a strength tester of salt-treated transmission poles (Friis-Hansen, 1980) and has potential for similar use in Australian conditions (Braid and Line, 1980; Leightley, 1981). Cown (1978) employed the device to assess the density of living trees in New Zealand, whilst Hedley and Naish (1980) successfully used it to examine the degradation of preservative-treated stakes.

Other possible techniques for the non-destructive testing of wood preservatives and fungal degradation of woods include the use of X-ray radiation for detecting rots in power-transmission poles (Gardner, Johnstone and Pitt, 1979), and assessing preservative performance in the laboratory (Thornton, Creffield and Collett, 1980). Gamma radiation has also been used to inspect power poles for degradation (Taylor, Morgan and Ellinger, 1980).

Kaiserlik (1978) examined some interesting alternative techniques for assessing 'soundness' of wood. These include stress wave average velocity, vibration (see also Wang, Suchsland and Hart, 1980) and acoustical emission and response spectra.

1.5.7 Laboratory Testing of Preservative Effectiveness

1.5.7.1 Physical assessment techniques

Methods involving weight loss of preservative-impregnated wood blocks following attack by wood-degrading fungi have been generally employed for the evaluation of wood preservatives in the laboratory. The soil-jar and decay chamber methods are useful when rapid and homogeneous decomposition is required (Duncan, 1960; Bravery, 1968; Henningsson, 1977). The treated wood blocks may be buried in -

(a) soil (Theden, 1961; Leightley and Russell, 1980) including sand (Hulme and Butcher, 1977). The test is severe and poorly reproducible (Cockroft, 1973; Gersonde and Kerner-Gang, 1976).

(b) vermiculite or similar artificial soil, if definition is required (Kaune, 1967; Gersonde and Kerner-Gang, 1976; Baines, Dickinson and Levy, 1977; Savory and Carey, 1980).

(c) agar inoculated with pure cultures of wood-rotting fungi (British Standard 838, 1961; Cockroft, 1973).

Assessment of the proportion of degraded wood in samples may be undertaken using microscopy. Ratings of wood degradation are commonly based on a pre-determined scale (e.g. 0 - no attack, 4 - heavily degraded sample) (Hoffmeyer, 1975; Henningsson, 1977).

Strength-testing of woods may be used to evaluate the degree of fungal attack. Techniques involving loss of resistance to impaction (Henningsson, 1977), crushing (Toole, 1971a), bending (Mateus, 1957) and tension (Brown, 1963) have been reported. A basic problem exists in the non-homogeneity of wood samples for these methods (Henningsson, 1977).

1.5.7.2 Biological assessment techniques

Bioassay techniques may be used for the rapid evaluation of wood preservative performance. These methods include the use of preservative-impregnated filter papers (Greaves, 1977b) or preservative-impregnated wood blocks (Scheffer and Gollob, 1978). Presence or absence of fungal growth, or inhibition of fungal growth are the general test criteria chosen with these techniques.

Biological techniques that may be used to assess preservative performance include estimates of fungal biomass in woods using chitin assays (Swift, 1973; Gurusiddaiah, Blanchette and Shaw, 1978) and respiration evaluation (Dovrtel and Schanel, 1974; Smith, 1975, 1976).

It is important that the laboratory assessment of wood preservatives is supported by subsequent field evaluation, since laboratory testing cannot duplicate the interactions of environmental conditions that challenge wood preservatives in the field (Beesley, 1978).

METHODS

2. METHODS

2.1 Wood Samples

Standing power transmission poles sampled were CCA-treated *Eucalyptus obliqua* or *E. globulus* hardwood species. Poles used for laboratory analysis were of the above species condemned because of soft-rot degradation at the ground-line. All poles examined had target retentions of 22kg/m^3 CCA.

Unless otherwise stated, sapwood samples taken from standing poles and pole stubs in the field were cores of 15mm diameter and 20mm depth. A 500W Makita drill with coring bit attached, powered by DC batteries or a Honda AC generator was used to obtain the samples. Where preservative-containing bandages were applied to pole stubs (2.12.1 + 2.12.2 + 2.12.3), a wad punch was utilized to cut a circular hole for sample procurement. The bandages were subsequently patched with heavy duty tape. All core holes were plugged with wood dowelling dipped in bituminous paint (Plate 3). Samples were transported to the laboratory in sealed plastic bags and stored at 2°C until use (within 7d).

Wood samples not requiring aseptic techniques were converted to a fine sawdust using a tennon saw before sieving through a 2mm mesh screen. Wood cores and samples required for isolation and enumeration of microorganisms were surface sterilised with absolute alcohol and sawdusted as before using aseptic techniques. The sawdust was collected in sterile 90mm petri dishes for subsequent culture.

Alternatively, poles removed from the ground were cut at the ground-line by a chain-saw. A further cut was made approximately 30mm below the ground-line level. The resultant

Plate 2 (top)

A section through the ground-line of a soft-rotted CCA-treated *Eucalyptus* sp. power transmission pole.

Plate 3 (lower)

A *Eucalyptus* sp. transmission pole after a core sample had been removed. Wood dowelling (15mm diameter) was used to seal the resultant plug-hole.

wood slivers were sectioned at intervals along the radial plane with a wood chisel. These sections were then sawdusted as detailed above.

2.2 Isolation and Enumeration of Microorganisms

Wood inhabiting microorganisms were isolated and enumerated (number of isolates per 0.01g sawdust) by plating sawdust samples directly onto agar media, using aseptic techniques. Fungi and bacteria were generally isolated for study from sawdust-inoculated swollen cellulose agar (2.5.1) and tryptic yeast extract agar (2.5.2 respectively).

2.3 Identification of Fungal Isolates

Fungal colonies were selected from Eucalyptus spp. wood sawdusts obtained from trial pole stubs inserted at Warrane, Tasmania, or from poles situated in northern and southern Tasmania.

The following criteria were considered important when identifying isolated Deuteromycetes (imperfect fungi) to species level: Method of conidia production; conidial size, shape, number of cells and pigmentation; presence or absence of slime; conidiophore morphology; appearance of fungal mycelium on agar media, including colour of fungal mycelium.

Additional characteristics used where applicable were noted by Hedgcock (1906), Raper and Thom (1949), Hesseltine (1954), Raper and Fennell (1965), Wang (1965), Sakapure and Thirumalacher (1966), Simmons (1967), Barron (1968), Toussoun and Nelson (1968), Schol-Schwarz (1970), Barnett and Hunter (1972) and Line and Cruickshank (1979).

No Ascomycetes were isolated in this study. Isolates thought to be Basidiomycetes were inspected for clamp connections but not further identified.

2.4 Identification of Bacterial Isolates

Randomly selected aerobic isolates from the ground-line of Eucalyptus spp. power transmission poles in Tasmania were assessed for the ability to degrade various substrates. Eleven isolates with proven cellulose or pectin-degrading capacities were identified to genus and where feasible to species level using Skerman's Key (Skerman, 1967) and Bergey's Manual of Determinative Bacteriology (1974). Bacillus sp. isolates were identified to species level using the additional aid of Gordon, Haynes and Pang's (1973) keys.

Bacteria were stained for endospore production using Schaeffer and Fulton's (1933) stain and for presence and production of flagella using Leifson's stain on 24h cultures. Tests involving growth in 7% NaCl, reduction of nitrate to nitrite, resistance to lysozyme, and growth in anaerobic agar were as described by Gordon, Haynes and Pang (1973). Ability of the isolates to hydrolyse starch was determined using the method outlined later in this study. The remaining biochemical tests used were described in Skerman (1967).

2.5 Media

Most media were sterilised at 121°C for 15 min. All glucose-containing media were sterilised at 108°C for 25 min.

2.5.1 Agar media for the cultivation of Fungi Imperfecti

(i) Glucose-asparagine agar [a modification of Martin's (1950) medium]: glucose, 15g, L-asparagine, 2.5g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 1g; yeast extract, 0.5g; agar, 15g; distilled water, 1L.

(ii) Swollen cellulose agar. A modification of Nilsson's (1973) BVII medium containing: $(NH_4)_2SO_4$, 0.5g; KH_2PO_4, 1g; KCl, 0.5g, $MgSO_4.7H_2O$, 0.2g; $CaCl_2.2H_2O$, 0.1g; yeast extract, 0.2g; agar, 15g; acid-swollen cellulose, 2.5g; distilled water, 1L. Whatman CC41 (W. & R. Balston, England) micro-granular cellulose powder was swollen with ortho-phosphoric acid according to the method of Walseth (1952). The swollen cellulose was dialysed with water (Union Carbide 50mm dialysis tubing) before repeated decantation and suspension.

(iii) Malt agar: Saunders (Victoria, Aust.) malt extract, 25g; agar, 20g; distilled water, 1L.

(iv) Copper sulphate agar (Starkey, 1973): glucose, 10g; $(NH_4)_2SO_4$, 0.5g; KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4$. $7H_2O$, 0.1g; $CaCl_2.2H_2O$, 0.1g; $FeSO_4.7H_2O$, 0.1g; agar, 15g; $CuSO_4.5H_2O$, 10g; distilled water, 1L. The copper sulphate was autoclaved separately in solution and added to the cooled medium.

2.5.2 Agar media for the cultivation of bacteria

(i) Tryptic yeast extract agar (TYE): BBL (Cockeysville, U.S.A.) trypticase soy broth, 3g; yeast extract, 1g; agar, 15g; nystatin, 0.05g; actidione, 0.05g; distilled water, 1L.

(ii) Carboxymethyl cellulose agar. A modification of the medium of Hankin and Anagnostakis (1977) was used, containing: KH_2PO_4, 4g; Na_2HPO_4, 6g; $(NH_4)_2SO_4$, 1g; $MgSO_4.7H_2O$, 0.2g; yeast extract, 1g; nystatin, 0.05g; actidione, 0.05g; Hercules (Wilmington, U.S.A.) 9M8F sodium carboxymethyl cellulose (NaCMC), 5g; agar, 10g; distilled water, 1L.

The following trace minerals were included: $FeSO_4.7H_2O$, 0.001g; $CaCl_2.2H_2O$, 0.001g; H_3BO_3, 10μg; $MnSO_4.H_2O$, 10μg; $ZnSO_4.7H_2O$, 70μg; $CuSO_4.5H_2O$, 50μg; MoO_3, 10μg. The final pH was 7.0. The NaCMC was suspended in the medium with a Waring blender.

2.5.3 Agar media for the cultivation of Basidiomycetes

Benomyl malt agar (Clubbe and Levy, 1977): Malt extract, 12.5g; agar, 15g; benomyl, 0.004g; streptomycin sulphate, 1g; distilled water, 1L. A stock solution of DuPont Chemicals (Wilmington, U.S.A.) benomyl ("Benlate") was prepared in 50% ethanol and added to the sterile, cooled agar.

2.5.4 Media for Enzyme Assays

2.5.4.1 Cellulases

2.5.4.1.a Media used for fungal Cx-cellulase production

(i) Microcrystalline cellulose medium. The modification of Nilsson's BVII medium described previously [2.5.1(ii)], was used with 1g/L $^{-1}$ Whatman CC41 cellulose replacing the swollen cellulose, and the agar omitted. Fifty ml of this medium was added to 250ml plugged and capped Erlenmeyer flasks. The tested pH was 5.4.

(ii) Crystalline cellulose (cotton wool) medium. The composition was as described above (i) except cotton wool, 50mg/30ml in 250ml Erlenmeyer flasks replaced microcrystalline cellulose.

(iii) Glucose medium. The composition was as described above (i) except 1gl^{-1} glucose substituted the microcrystalline cellulose. Each 250ml Erlenmeyer flask contained 50ml nutrient solution.

2.5.4.1.b **Medium used for bacterial Cx-cellulase production**

Modified Thayer and Murray's (1977) medium for the cultivation of cellulolytic bacteria was used, containing: $(NH_4)_2SO_4$, 1g; KH_2PO_4, 0.5g; K_2HPO_4, 1.5g; $MgSO_4.7H_2O$, 0.1g; $CaCl_2.2H_2O$, 0.01g; yeast extract, 0.5g; Whatman CC41 cellulose, 4g; distilled water, 1L. Trace minerals were included as detailed in 2.5.2.(ii). The tested pH was 6.8. Fifty ml of medium was added to 250ml Erlenmeyer flasks.

2.5.4.1.c **Media used for assay of cellulase production**

(i) **Solubilization of swollen cellulose agar** [Rautela and Cowling (1966) technique]. The basal substrate used for assessment of fungal clearing of swollen cellulose was described previously [2.5.1(ii)]. Five ml medium was added to 15mm diameter test tubes.

(ii) **Release of dye from Remazol Brilliant Blue R (RBBR)-dyed cellulose** (see 2.6.5.1 for cellulose-dyeing procedure). For fungi, dyed cellulose (2% final concentration) replaced the swollen cellulose in the modified Nilsson's medium [2.5.1.(ii)]. Double layers of this medium were poured in 15mm diameter test tubes (Smith, 1977), with the agar concentr-ation in the upper layer being 0.5% (0.5ml volume) and 0.75% in the lower layer (5.0ml). The lower layer lacked the dyed cellulose substrate.

Assessment of bacterial attack on dyed cellulose was by the same technique described for fungi, except the medium of Deschamps and Lebeault (1980) was utilized. This contained: $NH_4H_2PO_4$, 2g; KH_2PO_4, 0.6g; K_2HPO_4, 0.4g; $MgSO_4.7H_2O$, 0.5g; $CaCl_2.2H_2O$, 0.1g, $MnSO_4.H_2O$, 0.005g; yeast extract, 1g; distilled water, 1L.

2.5.4.2 Hemicellulases

The composition of the liquid media used for assessing fungal and bacterial xylanase production was as described earlier [2.5.4.1.a] and 2.5.4.1.b] but with larch (Larix sp., Sigma Chemicals, St. Louis, U.S.A.) xylan (0.2%) replacing cellulose. Five ml aliquots were added to 15mm diameter test tubes prior to autoclaving.

For the assessment of fungal clearing of hemicellulose agar deeps, additions of 0.125% *E. obliqua* xylan or 0.25% *E. obliqua* mannan (see 2.6.5.2. for extraction procedure from wood) were blended into modified Nilsson's (1973) BVII medium [2.5.1.(ii)] but without the cellulose added. Five ml aliquots were added to 15mm diameter test tubes prior to autoclaving.

The agar-well diffusion method of Stranks and Bienada (1971) was utilized for assessment of microbial xylanase production with larch xylan as the substrate. Double-layer plates were prepared with the base containing 1g agar and 0.005g sodium azide in 100ml 0.1M sodium acetate buffer (pH 5.5). The top layer contained the same ingredients but the agar concentration was reduced to 0.5% and 1% larch xylan was added.

2.5.4.3 Amylases

The composition of the liquid growth medium used for fungal amylase production was: $(NH_4)_2SO_4$, 0.5g; KH_2PO_4, 1g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.2g; $CaCl_2.2H_2O$, 0.1g; yeast extract, 0.5g; Univar (Sydney, Aust.) soluble starch, 2g; distilled water, 1L. Five ml medium was added to 15mm diameter test tubes prior to sterilisation.

The composition of the liquid medium used for bacterial amylase production was as detailed earlier [2.5.4.i.b] except $2gl^{-1}$ Univar soluble starch replaced the cellulose. Trace minerals described in 2.5.2.(ii) were also added. Five ml additions of solution were made to 15mm diameter test tubes.

The composition of the starch agar plates used for examination of bacterial amylase production was: Univar soluble starch, 0.2%, agar, 1.5%; sodium azide, 0.005%, in 0.1M phosphate buffer (pH 6.4).

2.5.4.4 Pectic Enzymes

The liquid growth medium employed for fungal pectic enzyme production had the following composition (R. Cruickshank, pers. comm.): ammonium tartrate, 10g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; yeast extract, 0.5g; citrus pectin, 2g; distilled water, 1L; pH 5.0. Five ml of medium was added to 15mm diameter test tubes prior to autoclaving. The tested pH was 5.7.

The composition of the medium used for bacterial pectic enzyme production was as detailed previously [2.5.4.1.b] with $1gl^{-1}$ citrus pectin replacing cellulose. The pH was 7.0. The trace minerals included in [2.5.2.(ii)] were added. Five ml of medium was added to 15mm diameter test tubes prior to autoclaving.

2.5.5 Media Used for the Assessment of Microbial Degradation of Eucalyptus obliqua sapwood

The composition of the low-nitrogen liquid growth medium in which fungal-inoculated sapwood blocks were incubated was: NH_4NO_3, 0.1g; glucose, 5g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.2g; yeast extract, 0.1g; distilled water, 1L.

For the examination of bacterial attack on *E. obliqua* sapwood, thin wood sections were saturated with Aaronson's (1970) medium comprising: $NaNO_3$, 1g; Na_2HPO_4, 1.2g; KH_2PO_4, 0.9g; $MgSO_4.7H_2O$, 0.5g; KCl, 0.5g; yeast extract, 0.5g; casein hydrolysate 0.5g; distilled water, 1L. The final pH was 6.4. Two wood slivers (10mm x 10mm x 20µm) were included with 2ml medium in 5ml Bijoux bottles prior to autoclaving.

2.5.6 Cultivation Medium for the Estimation of Fungal Chitin Contents

Fungal isolates were grown in 250ml Erlenmeyer flasks, each containing 40ml of the following low-nitrogen medium: NH_4NO_3, 0.1g; glucose, 0.5g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; yeast extract, 0.2g; distilled water, 1L.

2.6 Enzyme Assays

2.6.1 Assays for Cellulases

2.6.1.1 Determination of the Cx-cellulase activity of the microflora of wood samples

Unless otherwise stated, samples of sieved sawdust (2mm mesh) (0.2 or 0.3g) were incubated in 10ml of 0.4%NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 45C. The solutions were then filtered through tissue paper and either tested immediately for viscosity (using 1ml aliquots in a Wells-Brookfield microviscometer, Plate 18) or rapidly cooled to 0C to allow samples to be tested in batches. The viscometer was operated at 60 revs/min for 2 min prior to reading. The percentage reduction in viscosity was calculated using the formula:

$$\frac{C - S}{C - W} \times 100\%$$

where C = viscosity of NaCMC control or inactive sample,

S = viscosity of sample being assayed,

W = viscosity of water.

Either autoclaved sawdust samples, or more frequently samples treated with propylene oxide (Univar Chemicals, Sydney, Aust.) vapour for 36h (to denature Cx-cellulases), or unamended NaCMC solutions were used as controls.

To determine the effect of pH on Cx-cellulase activity, citrate-phosphate buffer [0.1M citric acid, 0.2M sodium phosphate (McIlvaine, 1921)] was utilized; effect of temperature on Cx-cellulase activity was determined using a temperature gradient incubator (Toyo Kagaku Sangyo TN-3).

2.6.1.2 <u>Determination of the Cx-cellulase activity of microbial culture filtrates</u>

Unless otherwise stated, the Cx-cellulase activity of culture filtrates was tested as described above (2.6.1.1), but using 1ml aliquots of filtrate incubated in 10ml of 0.5% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 50C. Unamended NaCMC solutions were used for controls. Culture filtrates were incubated for 10d at 22C before testing.

2.6.1.3 <u>Clearing of Walseth cellulose by fungal isolates</u>

Agar deeps [2.5.4.1.c] were stab-inoculated and incubated at 22C for 20d prior to examination of the depth of cellulose clearing.

2.6.1.4 <u>Release of dye from RBBR-dyed cellulose by microbial isolates</u>

The activity of microbial cellulases (if present) on the dyed cellulose in the upper layer of the tube resulted in release of dye into the lower layer (see Plates 20-21). Fungal-inoculated tubes were incubated for 10d at 22C before inspection whilst bacterial cellulase production was assessed after 30d incubation at 22C. Both fungal and bacteria-containing tubes were stab-inoculated.

2.6.1.5 Bacterial clearing of NaCMC agar

After inoculation, NaCMC agar plates (90mm diameter; see 2.5.2.(ii) for NaCMC agar composition) were incubated for 10d at 22C. Bacterial cellulase activity was determined by flooding the agar with 1% (w/v) aqueous hexadecyl trimethyl ammonium bromide (B.D.H. Chemicals, Poole, England) to precipitate the undegraded NaCMC.

2.6.2 Assays for Hemicellulases

After stab-inoculation and incubation for 30d at 22C, the tubes containing xylan or mannan were examined for depth of substrate clearing beneath the fungal mycelial mat at the surface.

For the assessment of microbial xylanase activity on larch xylan, wells of 9mm diameter were cut in the agar by a cork borer, and 0.05ml culture filtrate added to each. Fungal culture filtrates were incubated for 15d at 22C before testing, whilst bacterial filtrates had 10d growth at 22C. After 30h diffusion in the wells, the presence of clearing around each well was noted.

2.6.3 Assays for Amylases and Pectic Enzymes

Fungal and bacterial pectic enzyme and fungal amylase production were studied using polyacrylamide gel electrophoresis.

Methods for the detection of pectic enzymes in this study were described by Cruickshank and Wade (1980). Gels used for bacterial pectic enzyme detection were incubated after electrophoresis in 0.1M tartaric acid (buffer pH 2.0) or tris (hydroxymethyl) aminomethane/citric acid monohydrate buffer (pH 8.8; Cruickshank and Wade, 1980). Gels for the detection of amylases contained per 100ml gel buffer: Univar soluble starch, 0.15g; acrylamide, 7.5g; N,N'-methylene bisacrylamide, 0.25g; N,N',N"-tetramethylethylene diamine, 0.1ml; ammonium persulphate, 0.1g. All acrylamide reagents were supplied by B.D.H. Chemicals, Poole, England. The starch was dispersed in a minimal volume of buffer until the solution was clear. The electrophoresis and post-electrophoresis procedures for amylase detection were those detailed for pectic enzymes by Cruickshank and Wade (1980). Gels were stained after electrophoresis in a 1.5% KI/0.005% I_2 mixture for 24h.

Bacterial amylase production was assessed by an agar well diffusion method using starch agar plates. Wells of 9mm diameter were cut in the agar in petri plates. After addition of 0.05ml culture filtrate to the wells, the plates were left standing for 30h before staining with the above KI/I_2 mixture.

Fungal pectic enzymes and amylase productions were assessed after 20d and 12d culture incubations at 22C. Both bacterial amylase and pectic enzyme productions were examined after 10d at 22C.

2.6.4 Assay for Laccase Production

Fungi were grown on 2% malt agar for 7d at 22C. Bacterial isolates were cultured on TYE agar for 3d at 22C. The spot-test method of Harkin and Obst (1973) was used with a blue colour indicating laccase activity upon addition of drops of 0.1% alcoholic solution of syringaldazine [N,N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] (B.D.H. Chemicals, Poole, England).

2.6.5 Other Preparative Techniques for Enzyme Assays

2.6.5.1 Preparation of RBBR-dyed cellulose

The preparation of dyed cellulose was basically as described by Leisola, Linko and Karvonen (1975). A suspension of Whatman CC41 cellulose (100gl $^{-1}$ distilled water) was heated in a water bath at 50C with vigorous agitation. An equal volume of 1% aqueous Remazol Brilliant Blue R dye suspension (RBBR, Sigma Chemicals, St. Louis, U.S.A.) was added and the mixture agitated vigorously for 45 min with gradual addition of Na_2SO_4 (2g/g cellulose). A further addition of 10% aqueous Na_3PO_4 (1ml/g cellulose) followed with continuing agitation for a further 75 min. The dyed cellulose was recovered by filtration through muslin cloth. Hot tap water (60C) was added until the filtrate was colourless. The product was rinsed separately with acetone and ether and allowed to dry.

2.6.5.2 Extraction of hemicelluloses from Eucalyptus obliqua wood

Extraction of xylan and mannan from *E. obliqua* wood followed principles and variations detailed by Jermyn (1955), Whelan (1955), Meier (1958), Timell (1964), Dutton and Funnell (1973), Richards and Whistler (1973) and Bailey and Pickmere (1975).

A fine-powdered *E. obliqua* sawdust was obtained by milling splinters of sapwood in a Wiley mill. Sawdust samples were defatted with chloroform in a Soxhlet apparatus for 24h, dried at 60C and extracted with cold water to remove soluble tannins, proteinaceous material and arabinogalactans.

Sawdust samples were delignified as described by Holt and Gareth Jones (1978). Sawdust, (40g), $NaClO_2$ (18g) and 6ml of glacial CH_3COOH were added to 100ml water and heated to 55C. After 2h incubation, a further 3g $NaClO_2$ and 1ml glacial CH_3COOH were added. Following a further 2h heating at 55C, the sawdust was filtered, added to 2L of 0.5% ammonium oxalate and heated to 70C to depectinize the material.

The sawdust was washed with water and extracted with 300ml of 25% KOH under N_2 gas for 24h. Following adjustment of the pH to 5.0 with acetic acid, the material was filtered. Mannan was extracted from the residue whilst the filtrate was retained for extraction of xylan.

2.6.5.2.a Extraction of xylan

Xylan was precipitated from the filtrate by addition of absolute ethanol. Following filtration, the crude xylan was dried with a series of solvents of decreasing polarity (alcohol → petroleum ether). To improve the purity of the xylan, the precipitate was redissolved in 2% KOH to which Fehling's solution was gradually added until the copper complex was completely formed. The precipitate was filtered off and washed with 0.2N HCl to dissolve the copper complex. The xylan was dialysed with water (Union Carbide dialysis tubing, 50mm diameter) prior to centrifugation and drying over silica gel.

2.6.5.2.b Extraction of mannan

The *E. obliqua* residue remaining after the first alkali extraction was re-extracted under N_2 gas with 300ml of 17.5% NaOH containing 4% borate solution, and filtered. The filtrate was acidified to pH 5.0 with acetic acid and diluted 1:4 with saturated $Ba(OH)_2$ solution. Following centrifugation, the precipitate was washed with 0.2N HCl and dialysed with water for 24h, centrifuged and dried over silica gel.

2.6.6 Studies on the stability of Cx-cellulases following microbial death

To test various organic solvents for possible biocidal effects and denaturation of Cx-cellulases, sieved, soft-rotted wood sawdust was placed for 36h at 22-25C in 500ml screw-lidded bottles saturated with a range of solvent vapours. Vapour was removed by placing the opened bottles in a sterile air draught produced by a laminar flow for 3h. The sawdust samples were tested for viable microbial content by plating onto glucose-asparagine agar (for fungi) and tryptic yeast extract agar (for bacteria).

Samples (0.3g) were weighed into 15mm test tubes, vacuum evaporated to complete solvent removal, and then assessed for Cx-cellulase activity.

To determine the extent of Cx-cellulase leaching from wood, blocks of 100mm (parallel with the grain of the wood) x 15mm x 20mm dimensions were cut from soft-rotted sapwood of CCA-treated *Eucalyptus* sp. poles at the ground-line. After sterilization in chloroform vapour for 10d at 22C, 3 wood blocks were placed vertically in each of 8 jars containing either 30g vermiculite saturated with 270ml water, or dry vermiculite.

Aliquots of 1ml water surrounding the blocks in the 'wet' jars were tested for Cx-cellulase activity to estimate the leaching of enzyme from the wood. Checks were made on the sterility of the flask contents using sawdust agar plate counts. Additions of 0.005% sodium azide were made if any microbial growth was evident.

After incubation for 75d, the wood blocks were air-dried at 22C for 7d prior to sawdusting and testing for Cx-cellulase activity. A cutting depth of 5mm into the block sides containing the soft-rotted wood was made when sawdusting.

2.7 Fungal Degradation of *E. obliqua* sapwood blocks

E. obliqua sapwood blocks (either 20 x 20 x 20mm x 30 x 20 x 10mm) were air-dried, numbered, weighed and sterilised in propylene oxide vapour for 2d at 22C.

Screw-lidded glass jars of 500ml capacity were partly filled with 20g vermiculite amended with 70ml of low-nitrogen medium (2.5.5).

Four blocks were buried in each jar, leaving one side (parallel with the grain) exposed. The blocks were inoculated with agar pieces containing fungal mycelia. After 16 weeks incubation at 22C, the blocks were scraped free of adhering fungal mycelia, oven-dried for 24h at 100C and weighed. Determinations of weight changes of 12 replicate uninoculated blocks were used to estimate the weight losses of the inoculated blocks.

2.8 Embedding and Sectioning of Hardwoods for Microscopic Inspection

Eucalyptus obliqua wood sections (10mm x 10mm x 20 μm), cut by a Leitz Wetzlar base-sledge microtome and subjected to bacterial attack, or slivers of soft-rotted Eucalyptus sp. sapwood were evacuated in a vacuum desiccator, fixed overnight in 5% glutaraldehyde (in 0.1M phosphate buffer, pH 7.3) and dehydrated through a series of alcohol concentrations (0%-100% alcohol) followed by propylene oxide, with 30 min at each stage. Specimens were embedded in Spurr's (1969) resin or Araldite (Appendix 1). If embedded in Spurr's resin, the specimens were impregnated with a 50% mixture of resin in absolute alcohol for 24h prior to placing in Ladd silicone rubber moulds and polymerizing in pure resin at 70C for 18h. The resin was pared from one wood face and the specimens softened using the method of Alcorn and Ark (1952) before sectioning with an LKB Ultratome microtome.

Araldite-embedded sections were polymerized for 4d at 65C before sectioning as described above. Wood sections were stained with crystal violet (Skerman, 1967) before examination with a Zeiss standard RA microscope.

2.9 Scanning Electron Microscopy (SEM)

Thin sections of wood were fixed in 1% OsO_4 in 0.1M phosphate buffer, pH 7.2 for 14d before water-washing and dehydration through an alcohol series followed by petroleum ether. The specimens were finally dried over silica gel, mounted on copper stubs and gold-coated with a Dynavac sputter coater Model SC150 (20mA for 3 min at 0.3 Torr). The sections were examined with a Jeol JX50U SEM.

2.10 Chitin Assays

2.10.1 The Alkaline Deacetylation Technique

The method used was similar to that of Ride and

Drysdale (1972). Samples (0.2g) of sieved sawdust were homogenised twice with acetone in a Sorvall omnimixer with microcup attachment. After centrifugation (4000g for 10 min at 4C) and removal of the supernatant, the samples were washed with water and recentrifuged. Aliquots (5ml) of 120% KOH were added to the tubes containing the residue and heated for 1h at 130C. To each tube, 8ml volumes of 75% ethanol were added, and tubes left standing in ice water for 15 min. A 0.9ml volume of Celite solution (Sigma Chemicals, St. Louis, U.S.A.) (1g Celite in 20ml of 75% alcohol, left standing for 2 min prior to use) was layered on top of each tube prior to centrifugation (4000g for 10 min at 4C). The supernatant was removed and the residue washed with 40% ethanol and recentrifuged as above. The residue was washed twice more with distilled water.

Equal volumes of 1.5ml chitosan suspension, 5% $NaNO_2$ and 5% $KHSO_4$ were transferred to a centrifuge tube, shaken for 15 min and centrifuged (4000g for 10 min at 4C) before 2 x 1.5ml samples of supernatant were removed for assay. To each sample, 0.5ml of 12.5% ammonium sulphamate ($NH_4SO_3NH_2$, Sigma Chemicals, St. Louis, U.S.A.) was added. After shaking for 15 min, 0.5ml additions of 3-methyl 2-benzothiazolone hydrazone hydrochloride (MBTH) (Sigma Chemicals, St. Louis, U.S.A.) were made. The solutions were heated in a boiling water bath for 3 min, cooled and 0.5ml of 0.5% $FeCl_3$ added. After standing for 30 min, the optical density at 650nm was read in 10mm cells. Appropriate glucosamine (B.D.H. Ltd., Poole, England) standards were included.

2.10.2 The Acid Hydrolysis Technique

Unless otherwise stated, samples of sawdusted wood (0.2g), dried fungal mycelia of known weight (0.01-0.10g) or purified chitin (0.02g) were hydrolysed in 5ml of 5N HCl for 20h at 80C in stoppered Exelo C19 Quickfit tubes. Purified chitin was obtained either from Sigma Chemicals (St. Louis, U.S.A.) or purified from crab shell chitin by the method of Skujins, Potgieter and Alexander (1965).

After hydrolysis, samples were filtered (Whatman No. 41 paper) and 1ml aliquots diluted with 5ml water. Aliquots of 2ml volume were then added to glass columns (600 x 5mm, Plate 19) containing Dowex-50W (Sigma Chemicals, St. Louis, U.S.A.) strongly-acid cation exchange resin prepared exactly as described by Boas (1953). After washing with distilled water, the glucosamine was eluted into 5ml volumetric flasks with 2N HCl.

The solutions were transferred to test tubes. One drop of 0.1% methyl red indicator in alcoholic solution was added to each tube, followed by drops of 100% KOH solution until a yellow colour appeared. Concentrated HCl was then added until the red colour just reappeared.

Colorimetric reagents were added to 1ml samples placed in fresh tubes, basically as reported by Tsuji, Kinoshita and Hoshino (1969). To each of these tubes, 1ml of 5% $KHSO_4$ and 1ml of 5% $NaNO_2$ were added and the tubes left with occasional shaking for 15 min. This was followed by additions of 1ml aliquots of 12.5% $NH_4SO_3NH_2$ and after a further 5 min shaking, 1ml aliquots of freshly prepared 0.5% MBTH solution were added. The mixtures were allowed to stand for

60 min at room temperature before colour production was induced by additions of 1ml volumes of 0.5% $FeCl_3$. The solutions were left standing in the tubes for a further 10 min before absorbances were read against a reagent blank at 630nm using a BBL Spectronic 20 spectrophotometer.

Absorbances at varying wavelengths were scanned using a Unicam SP800 spectrophotometer.

Fungal cultures for chitin content determinations were harvested and washed in sintered glass funnels before oven-drying at 100C to constant weight.

Activated charcoal was supplied by M & B Chemicals, Dagenham, England, whilst polyvinyl polypyrrolidone was supplied by Sigma Chemicals, St. Louis, U.S.A. Most amino acids were supplied by Calbiochem, San Diego, U.S.A.

2.11 Direct Techniques for the Assessment of Wood Degradation

2.11.1 Microscopic assessment of cell wall degradation of woods

Wood samples were boiled in a 1:1 mixture of glycerol and water for 30 min (Henningsson, 1977) prior to sectioning with a razor blade. Sections were stained with saffranine solution (glycerol, 25ml; 70% ethanol, 25ml; saffranine, 0.1g; distilled water, 50ml) and examined microscopically. The percentage cell wall degradation was estimated by comparison with reference sections.

2.11.2 Visual inspection of wood degradation

Sapwood stakes emplaced at Grove, Tasmania, were inspected and ranked according to their condition at the ground-line after incubation in the soil. A sharp knife was used to detect decay which was categorised as follows:

- a completely rotted
- b large pockets of rot, very soft
- c pockets of rot in some areas
- d trace of softening in isolated pockets
- e sound, very hard to knife in all areas of stakes.

2.11.3 Use of the Pilodyn (R) Impaction Instrument

Standing poles (and standing pole stubs) were tested at the ground-line, with 4 determinations of pin penetration per pole being averaged. Both 6J (2mm pin diameter) and 10J (2.5mm pin diameter) Pilodynes (R) were used.

2.12 Field Trials of Wood Preservatives

2.12.1 Evaluation of Assay Techniques for the Assessment of Wood Degradation using a Pole Stub Trial at Grafton, N.S.W. (Koppers Aust. Pty. Ltd.)

Details of the preservative treatments relative to the present investigation are included in Appendix 5 .

As part of Koppers Pty. Ltd. trial of pre-applied barriers to soft-rot attack at the ground-line, 27 *Eucalyptus maculata* pole stubs (2 untreated, 5 CCA-treated and 20 CCA + additional treatment) were tested by the Cx-cellulase assay (1 sample core per stub) and impaction determinations with the 6J Pilodyne (R). The stubs were in position for 2 years at the time of testing. The site has a mean annual rainfall of 983mm.

2.12.2 Evaluation of Biological Assay Techniques for the Assessment of Wood Degradation using a Pole Stub Trial at Warrane, Tasmania

This trial was jointly devised and erected by the

University of Tasmania and the Hydro-Electric Commission of Tasmania. The site chosen was on land fill (Appendix 11), with a mean annual rainfall of 630mm.

Twenty _Eucalyptus globulus_ (possibly some _E. obliqua_) poles of aged but fair condition were cut into 1.8m sections with the bottom portion previously in ground contact being discarded. Sections were checked for excessive fungal or bacterial colonization prior to installation (M.A. Line, unpublished information). They were emplaced 2m apart in January 1978, and top-capped with heavy-duty aluminium foil. A water-trickle system of irrigation was provided at the base of each stub.

The predominant soft-rot fungi isolated in a survey of Tasmanian poles (Line and Cruickshank, 1979) were separately cultivated in nutrient-amended sawdust medium mixed and evenly distributed around the base of each pole (Line, pers. comm.). Three further supplements of sawdust plus ammonium sulphate were made to the site in an attempt to encourage soft-rot attack.

Five further stubs were added to the trial 6 months after the installation of the original stubs to act as untreated controls (i.e. without addition of preservatives). Details of the trial layout, preservative formulations and methods of application are given in Appendix 7 and Plates 22-28.

Remedial wood preservatives were applied to the groundline of the stubs in February, 1979, 13 months after stub emplacement. Five replicate stubs were used for each treatment. The pole stubs were sampled 12 months after emplacement but just prior to application of remedial preservatives [January, 1979; Sample (i)], 6 months after application of preservatives

[Sample (ii)], and 18 months later [Sample (iii)]. Two cores per stub were extracted in Samples (i) and (ii), whilst 5 cores per stub were removed and analysed in Sample (iii).

Preservative performance was assessed using the fungal propagule count technique and the Cx-cellulase assay.

2.12.3 Evaluation of Biological Assay Techniques for the Assessment of Wood Degradation using a Pole Stub Trial at Coffs Harbour, N.S.W. (New South Wales Forestry Commission)

This trial of wood preservatives for ground-line maintenance treatments was begun in 1976 and has been described by Johnstone, Gardner and Pitt (1979) (Plate 29). The site has a mean annual rainfall of approximately 1050mm. Preservative treatments were installed at the ground-line soon after stub emplacement. The CSIRO-developed heat-shrink bandages [Mark IV, cross-linked polyethylene (XLPE) backing] were installed on stubs 3 years later as an additional trial.

Four years after the insertion of the main trial, core samples (20mm diameter, 50mm depth) were forwarded for comparative evaluation using the fungal propagule count and the Cx-cellulase assay.

2.12.4 Evaluation of Assay Techniques for the Assessment of Wood Degradation using a Eucalyptus obliqua Sapwood Stake Trial at Grove, Tasmania

The plan and other details of this trial are given in Appendices 9 and 11, and Plates 30-34. The mean annual rainfall of the site is 905mm.

Sapwood from 12-year-old *E. obliqua* saplings (200-250mm ground-line diameter) were cut to obtain 100 sapwood stakes of 300 x 40 x 15mm dimensions. The stakes were dried by force-draught ventilation at 70C for 12h.

Preservative treatments applied were

(i) Creosote [hot and cold bath, Findlay (1962)]. Stakes were heated to 90C for 45 min and left to cool for 72h at ambient temperatures (10-15C).

(ii) Tanalith C paste [Koppers Pty. Ltd., Sydney (hot and cold bath)]. Approximate salt composition $K_2Cr_2O_7$, 45%, $CuSO_4.5H_2O$, 35%; $As_2O_5.2H_2O$, 20%. Stakes were boiled for 45 min in the paste and left standing for 48h fully immersed. The treatment was completed by forced-draught drying at 70C for 12h.

(iii) $CuSO_4.5H_2O/K_2Cr_2O_4$ (hot and cold bath). Stakes were boiled in a 5% (w/v) solution of $CuSO_4.5H_2O$ for 30 min and left to cool at ambient temperature for 24h. After 72h fixation in 1% K_2CrO_4, they were forced-draught dried at 70C for 12h.

(iv) $H_3BO_3/Na_2B_4O_7.10H_2O$ (hot and cold bath). A 5% solution of boric acid was neutralized with 1:1.54 parts of decahydrate borax (Findlay, 1962). The stakes were boiled in this solution for 45 min, left to cool at ambient temperature for 24h fully immersed, then forced-draught dried at 70C for 24h.

(v) Sodium pentachlorophenate (NaPCP). The stakes were placed in a saturated solution containing 500g NaPCP flakes in 2L mineral turpentine for 24h at 22C.

(vi) Bituminous paint. One half of each stake was evenly brush-coated with Pabco bituminous paint to a thickness of ca. 3mm.

(vii) Untreated controls.

Where applicable, preservative retentions (calculated on a wood density basis) are listed in Appendix 9.

Eight stakes per treatment were randomly placed in a grid pattern (Appendix 9) for 9 months exposure prior to inspection and assay.

The techniques used to assess microbial attack of the stakes were visual appraisal of wood condition, agar plate counts for fungi and bacteria, Cx-cellulase assays and chitin assays (for fungal biomass estimates).

RESULTS

3. RESULTS

3.1. Fungi

3.1.1 Predominant Fungi Isolated from Tasmanian CCA-Treated Eucalyptus sp. Hardwood Poles, and Other Woods Examined

The predominant fungus isolated from CCA-treated eucalypt poles in this study was *Phialophora mutabilis*. This organism was present in most samples of soft-rotted wood examined from locations around Tasmania. It was also frequently isolated from treated *Eucalyptus maculata* and *Pinus radiata* pole stubs situated at Coff's Harbour, N.S.W. (Appendix 8) and intermittently from samples of treated *E. maculata* stubs at Grafton, N.S.W. (Appendix 5). *Phialophora mutabilis* was prevalent in samples from both treated and untreated *Eucalyptus obliqua* stakes emplaced at Grove, Tasmania.

Other fungi commonly isolated from CCA-treated woods in this study were *Paecilomyces varioti*, *Fusarium* spp., *Cephalosporium acremonium*, *Alternaria* spp., *Oidiodendron griseum*. *Graphium ridigum* and various *Penicillium* spp. *Ambylosporium* sp. was commonly found only in woods examined from the northern and north-western areas of Tasmania.

In the time studied, *Trichoderma viride* was the predominant fungus isolated from both untreated and treated *E. obliqua* stakes emplaced at Grove, Tasmania. In addition, this organism was often isolated from many CCA-treated hardwood poles examined in the course of this study.

3.1.2 Aspects of the Enzymology of Wood-Inhabiting Imperfect Fungi Isolated from Tasmanian CCA-Treated Hardwood Poles

The ability of selected fungal isolates forming the

<u>Some microfungi isolated from the ground-line of Tasmanian soft-rotted CCA-treated *Eucalyptus* sp. transmission poles</u>

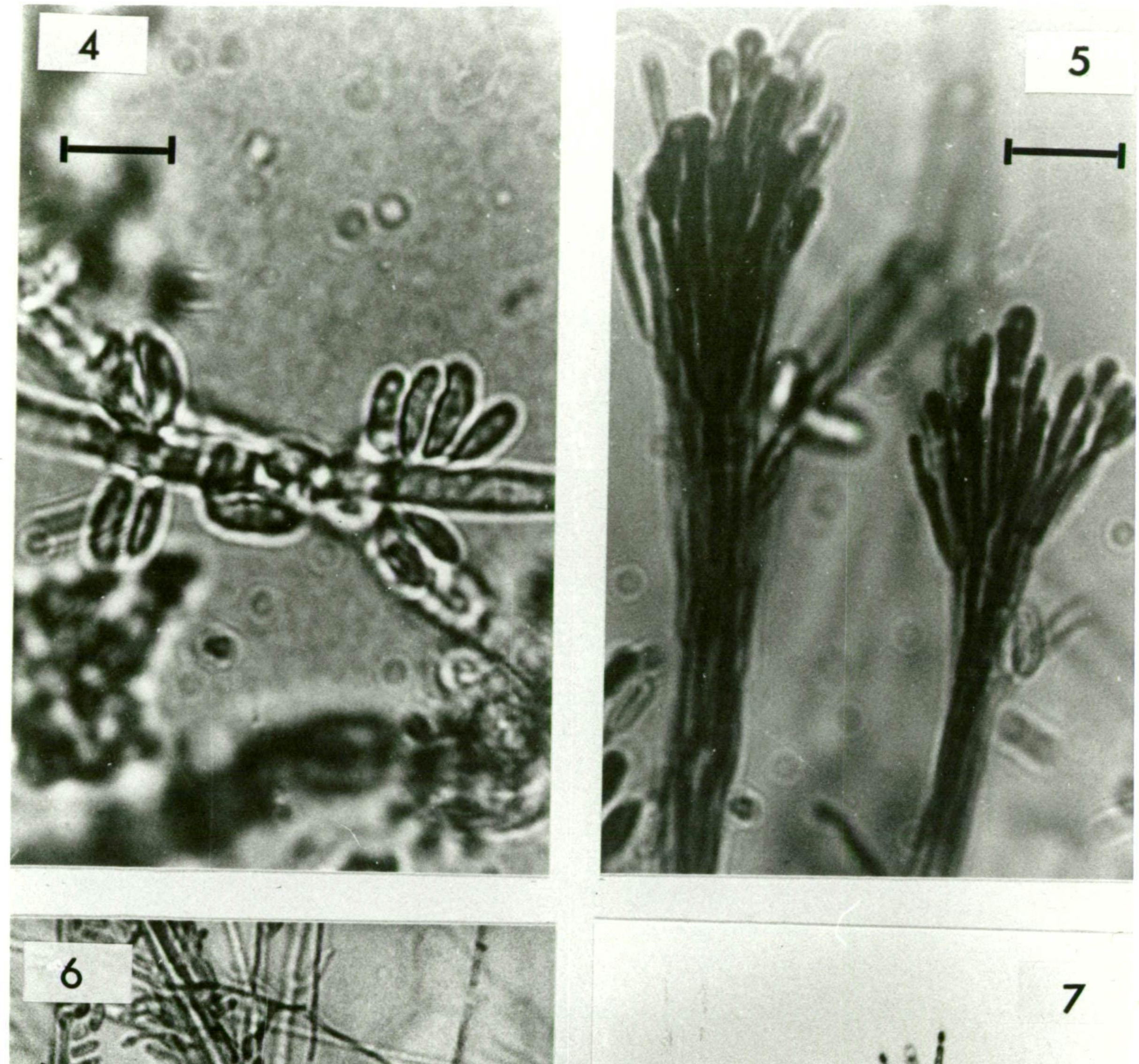
The fungi were photographed on glucose-asparagine agar, using a Zeiss standard RA Microscope.

Plate 4. *Aureobasidium pullulans*. Bar = 7µm.

Plate 5. *Graphium rigidum*. Bar = 10µm.

Plate 6. *Phialophora mutabilis*. Bar = 15µm.

Plate 7. *Paecilomyces varioti*. Bar = 25µm.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predominant mycoflora of soft-rotted wood to elaborate enzymes for the degradation of wood substrates was assessed using a variety of assay procedures.

3.1.2.1 Production of Cellulases by Fungi

Most of the fungal isolates examined could produce Cx-cellulase (EC 3.2.1.4) when grown in liquid cellulose-containing media (Table 1). Aureobasidium pullulans, Paecilomyces varioti and Septonema sp. were, however, not capable of producing Cx-cellulase under the conditions used. Assay for Cx-cellulase activity in a medium lacking cellulose showed inducible production in Phialophora mutabilis (strain B), Cephalosporium acremonium, Doratomyces microsporus, Polystictus versicolor (a white-rotting Basidiomycete included for comparison) and probably Graphium rigidum. Constitutive producers were Phialophora mutabilis (strain A), Aspergillus fumigatus, Chaetomium globosum, Fusarium decemcellulare, Penicillium frequentans and Pyrenochaeta sp.

Of the isolates tested, only Paecilomyces varioti, Aureobasidium pullulans and Septonema sp. could not degrade swollen cellulose or release dye from RBBR-microcrystalline cellulose (Table 1). The remaining isolates generally had pronounced cellulolytic activity when assessed by both techniques. The Basidiomycete Polystictus versicolor caused considerable solubilization of the swollen cellulose but released minimal dye from RBBR-cellulose, whilst Chaetomium globosum and Graphium rigidum produced the reverse effect. Fusarium decemcellulare, Phialophora mutabilis (strain B) and Trichoderma viride had high levels of cellulase activity when assessed by all three techniques.

Table 1.

Evaluation of enzyme activity on cellulosic substrates by fungi isolated from Tasmanian CCA-treated hardwood poles

Fungal Isolate	Percent reduction in viscosity NaCMC after growth in substrates: 0.1% glucose	0.1% micro-crystalline cellulose	Cotton wool medium	RBBR - cellulose dye release	Depth clearing (mm) cellulose agar deeps
Aspergillus fumigatus	10.3	75.1	68.1	++	9(C)
Aureobasidium pullulans	ns	ns	ns	ns	ns
Cephalosporium acremonium	ns	49.6	33.1	++	4(C)
Chateomium globosum	9.7	65.5	88.2	+++	3(C)
Doratomyces microsporus	ns	6.1	23.2	++	3(C)
Fusarium decemcellulare	5.7	83.2	90.2	+++	12(C)
Graphium rigidum	ns	ns	75.6	+++	2(C)
Oidiodendron griseum	ns	ns	46.9	+	5(C)
Paecilomyces varioti	ns	ns	ns	ns	ns
Penicillium frequentans	8.6	8.0	44.9	+	9(C)
Phialophora mutabilis (Strain A)	13.1	82.7	80.3	++	3(C)
Phialophora mutabilis (Strain B)	ns	74.3	71.3	+++	12(C)
*Polystictus versicolor	ns	66.8	39.8	+	17(D)
Pyrenochaeta sp.	10.3	80.5	41.7	++	11(D)
Septonema sp.	ns	ns	ns	ns	ns
Trichoderma viride	ns	90.7	61.8	+++	9(C)

*White rotting Basidiomycete included for comparison.

(C) - Sharp Clearing; (D) Diffuse Clearing

+++ - strong colour production

++ - moderate colour production

+ - weak colour production

ns = non-significant level of activity

Cx-cellulase assay values were means of duplicate determinations. Aliquots (1ml) of culture filtrate (10d growth at 22°C) were incubated with 10ml 0.5% NaCMC in acetate buffer (pH 5.5) for 1h at 50°C.

Values obtained from agar (Rautela and Cowling) deeps were means of duplicate determinations. The tubes were incubated for 20d at 22°C.

RBBR-cellulose dye release estimates were obtained from duplicate tubes. The incubation period was 10d at 22°C.

Most fungi produced a sharp clearing of the swollen cellulose. However, *Polystictus versicolor* and *Pyrenochaeta* sp. formed a diffuse boundary between the cleared and uncleared cellulose.

3.1.2.2 Production of Hemicellulases by Fungi

Eucalyptus obliqua xylan and mannan, and larch (*Larix* sp.) xylan were all degraded by isolates of *Alternaria* sp., *Chaetomium globosum*, *Cephalosporium acremonium* and *Trichoderma viride* (Table 2). *E. obliqua* mannan, and to a lesser extent larch xylan were degraded by most of the fungi. All isolates grew vigorously on *E. obliqua* xylan, but zones of clearing in agar media were rarely observed. The *E. obliqua* mannan appeared to be the most readily utilizable substrate of those under test.

3.1.2.3 Production of Amylases by Fungi

Of the 16 fungal isolates tested, 13 were capable of amylase production as determined by polyacrylamide gel electrophoresis (Table 3). Based on differences in migration (Rf) distance (Plate 10), *Aspergillus fumigatus* produced between five and seven distinct amylases, whilst *Graphium rigidum*, *Aureobasidium pullulans*, *Cephalosporium acremonium*, *Penicillium frequentans*, *Polystictus versicolor* and *Trichoderma viride* all produced more than one detectable enzyme. Little similarity was observed between enzymes produced by different fungi when Rf values were compared.

3.1.2.4 Production of Pectic Enzymes by Fungi

Of the isolates tested in this study, all except *Cephalosporium acremonium*, *Fusarium decemcellulare*, *Penicillium*

Table 2.

Evaluation of hemicellulase production by fungi isolated from Tasmanian CCA-treated hardwood poles.

Fungal Isolate	Substrate		
	Larch (*Larix* sp.) xylan	*E. obliqua* xylan	*E. obliqua* mannan
Alternaria sp.	+	+	+
Ambylosporium sp.	-	-	NT
Aspergillus fumigatus	+	-	+++
Cephalosporium acremonium	+	+	++
Cladosporium herbarum	NT	-	NT
Chaetomium globosum	+	+	+
Doratomyces microsporus	+	-	+
Graphium rigidum	-	+	+++
Humicola sp.	NT	-	NT
Oidiodendron griseum	+	-	++
Paecilomyces varioti	-	-	+
Penicillium frequentans	++	-	+
Phialophora mutabilis (Strain A)	++	-	+++
Pyrenochaeta sp.	+	-	-
Trichoderma viride	++	+	+++
Volutella sp.	NT	-	-

- = negative, no activity detected

+ - weak activity (less than 4mm agar clearing)

++ - moderate activity (4-8mm)

+++ - strong activity (greater than 8mm)

Activities on *Eucalyptus obliqua* hemicelluloses were determined using agar deeps after 30d growth at 22 °C, whilst degradation of larch xylan was assessed by the agar-well diffusion technique. Cultures were grown for 15d at 22° C before testing. Aliquots (0.1ml) were added to the agar wells which were left at 22° C for 30h before inspection.

Activity on *E. obliqua* xylan: (+)ve xylanase producers formed visible clearing zones at base of fungal mycelia.

frequentans, *Graphium rigidum*, *Paecilomyces varioti* and *Septonema* sp. produced detectable pectic enzymes when examined by polyacrylamide gel electrophoresis (Table 3).

Most isolates produced polygalacturonases (EC 3.2.1.5) as the sole enzyme form, but *Oidiodendron griseum* and *Polystictus versicolor* exhibited measurable pectin esterase (EC 3.1.1.11) as well (Plate 11). Pectin lyase (EC 4.2.2.2) production by fungi was not examined.

3.1.2.5 Production of Laccases by Fungi

Seven fungal species were tested for laccase production (EC 1.10.3.2) activity by the method of Harkin and Obst (1973). Positive results were obtained for *Graphium rigidum*, *Cephalosporium acremonium* and *Fusarium decemcellulare*, whilst tests for production by *Chaetomium globosum*, *Oidiodendron griseum*, *Phialophora mutabilis* (strain A) and *Penicillium frequentans* were negative.

3.1.3 Fungal Degradation of *Eucalyptus obliqua* Sapwood Blocks

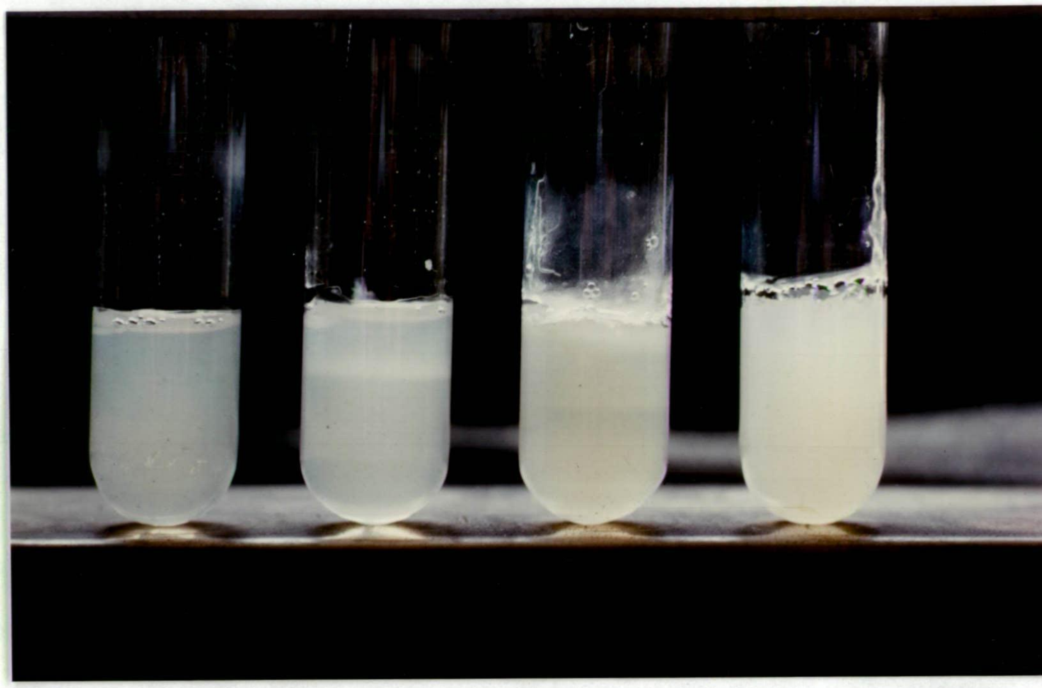
The comparative abilities of selected fungi to degrade *E. obliqua* sapwood was assessed by the weight losses of small sapwood blocks. After 16 weeks incubation at 22C, most fungal inoculated blocks had a resultant small weight loss (Table 4). In comparison with the white-rotting Basidiomycete *Polystictus versicolor*, the weight loss of blocks inoculated with imperfect fungi was low. *Pyrenochaeta* sp. (2.95-5.65% weight loss), *Doratomyces microsporus* (2.13-4.54%), *Graphium rigidum* (3.86-4.33%) and *Phialophora mutabilis* (strain A) (2.54-3.58%) produced the highest weight losses of the imperfect fungi tested.

Plate 8

Clearing of Eucalyptus obliqua hemicelluloses by Tasmanian fungal isolates. From left to right the test tubes contained: E. obliqua xylan, (uninoculated control); xylan plus Trichoderma viride; mannan plus Penicillium frequentans; uninoculated mannan. After inoculation, the tubes were grown for 15d at 22^{o}C before examination.

Plate 9

Clearing of starch agar by bacterial culture filtrates. Clockwise from top-left the agar wells contained culture filtrates of: Bacillus carotarum (strain 1); Bacillus carotarum (strain 2); Flavobacterium ferrugineum; Nocardia rugosa and Bacillus megaterium (in the middle well). Cultures were grown for 10d at 22^{o} C before assessment. Aliquots (0.1ml) were added to the agar wells.



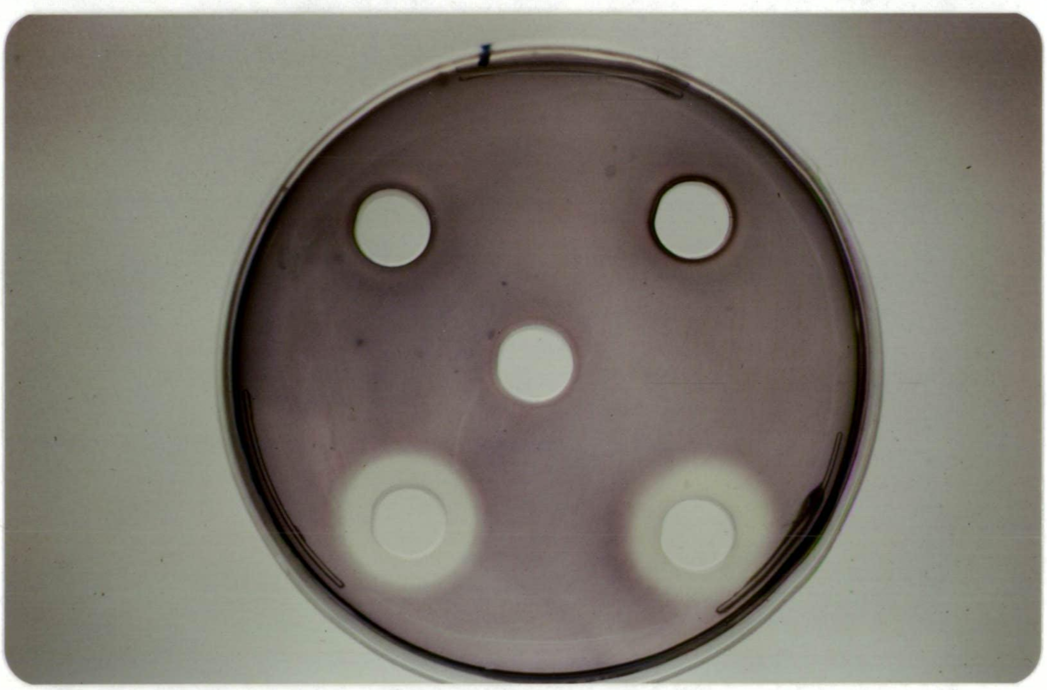


Table 3.

Evaluation of amylase and pectic enzyme production by fungi isolated from Tasmanian CCA-treated hardwood poles using polyacrylamide gel electrophoresis.

Fungal Isolate	Amylase activity	*Pectic enzyme activity
Aspergillus fumigatus	+ (5-7)	+ (3)
Aureobasidium pullulans	+ (2)	+ (2)
Cephalosporium acremonium	+ (2)	-
Chaetomium globosum	+ (1)	+ (1)
Doratomyces microsporus	+ (1)	+ (1)
Fusarium decemcellulare	+ (1)	-
Graphium rigidum	+ (2)	-
Oidiodendron griseum	-	+ (3) PE (1)
Paecilomyces varioti	-	-
Penicillium frequentans	+ (2)	-
Phialophora mutabilis (Strain A)	+ (1)	+ 2
Phialophora mutabilis (Strain B)	+ (1)	+ 3
Polystictus versicolor	+ (2)	+ (6-10) PE (1)
Pyrenochaeta sp.	+ (1)	+ (3)
Septonema sp.	-	-
Trichoderma viride	+ (2)	+ (1)

( ) = minimum number of enzymes observed.

PE = pectin esterase.

*All pectic enzymes observed were polygalacturonases (EC 3.2.1.11.) unless otherwise stated.

Amylase activity was determined after 12d culture growth at 22°C. Pectic enzyme production was tested after 20d incubation at 22°C.

Plate 10

Detection of fungal amylase production by polyacrylamide gel electrophoresis. Well No. 1 is on the far left.

Well

1. *Trichoderma viride*
2. *Doratomyces microsporus*
3. *Paecilomyces varioti*
4. *Oidiodendron griseum*
5. *Chaetomium globosum*
6. Control
7. *Pyrenochaeta* sp.
8. *Septonema* sp.
9. *Aureobasidium pullulans*
10. *Cephalosporium acremonium*
11. Control
12. *Polystictus versicolor*
13. Control
14. *Phialophora mutabilis* (Strain B)
15. *Penicillium frequentans*
16. *Phialophora mutabilis* (Strain A)
17. *Aspergillus fumigatus*
18. *Fusarium decemcellulare*
19. *Graphium rigidum*
20. *Cladosporium herbarum*

Amylase activity was determined after 12d culture growth at 22^{o}C.

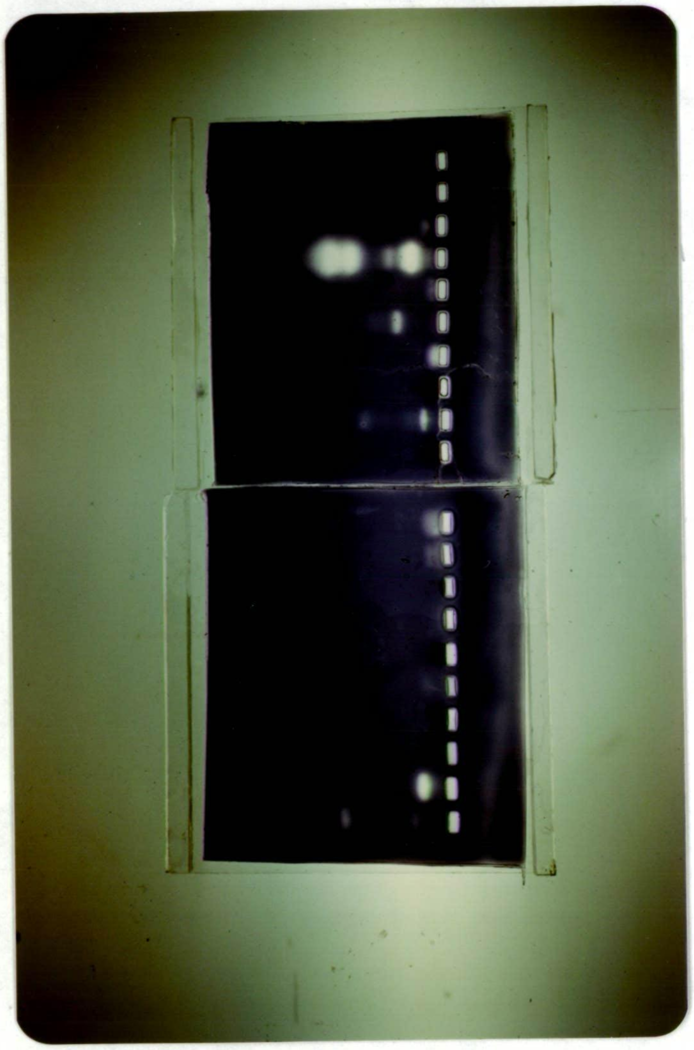


Plate 11

Detection of fungal pectic enzyme production by polyacrylamide-gel electrophoresis. Well No. 1 is on the left of the plate.

Well

1. *Trichoderma viride*
2. *Doratomyces microsporus*
3. *Paecilomyces varioti*
4. *Oidiodendron griseum*
5. *Chaetomium globosum*
6. Control
7. *Pyrenochaeta* sp.
8. *Septonema* sp.
9. *Aureobasidium pullulans*
10. *Cephalosporium acremonium*
11. *Phialophora mutabilis* (Strain B)
12. *Graphium rigidum*
13. *Fusarium decemcellulare*
14. *Aspergillus fumigatus*
15. *Phialophora mutabilis* (Strain A)
16. *Penicillium frequentans*
17. *Cladosporium herbarum*
18. Control
19. *Polystictus versicolor*

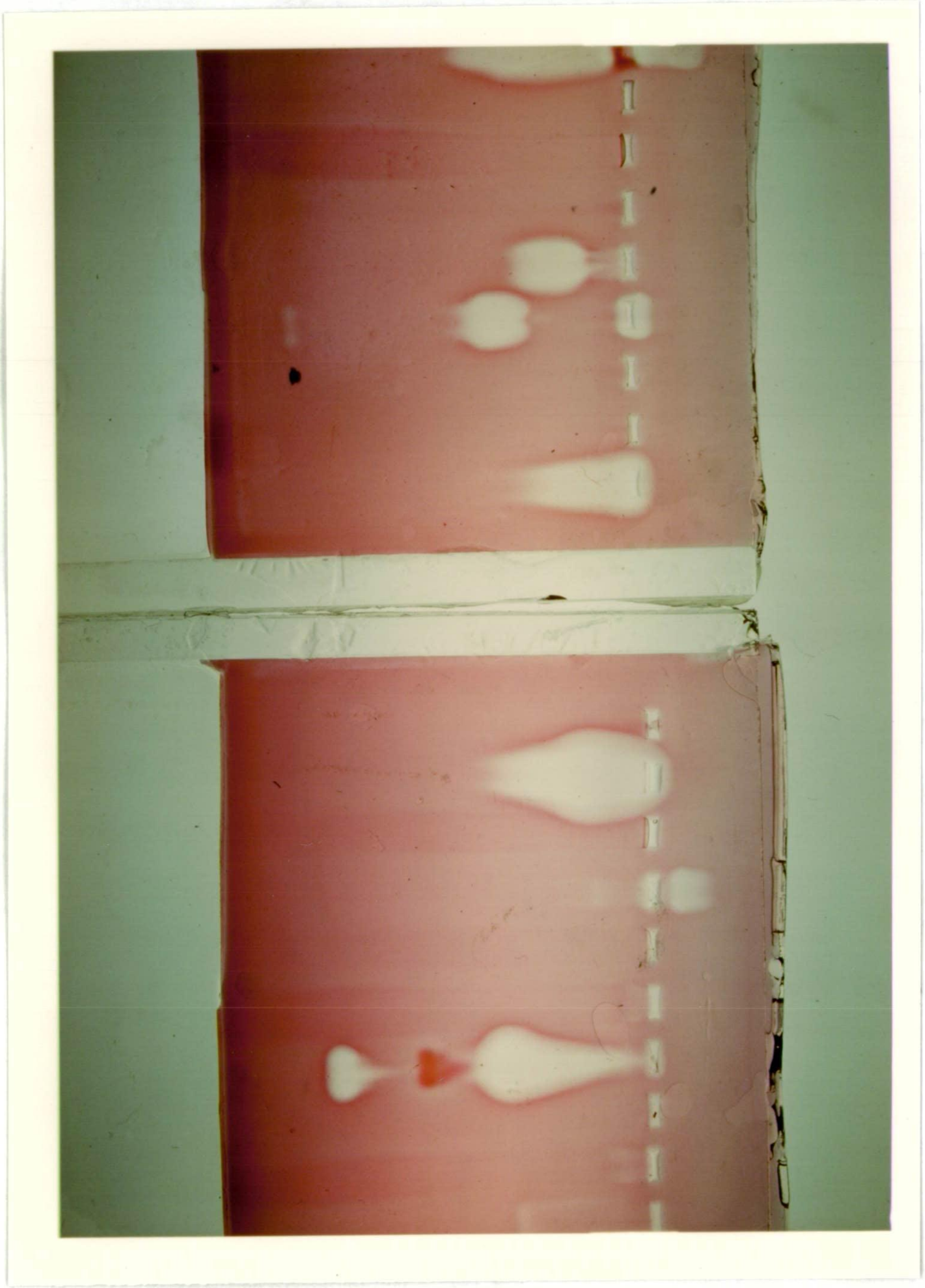


Table 4.

Degradation (weight loss) of Eucalyptus obliqua sapwood blocks by fungi isolated from Tasmanian CCA-treated wood poles.

Fungal Isolate	Percent weight loss (Experiment 1)		Percent weight loss (Experiment 2)	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.
Alternaria sp.	0.80	0.29	4.13	0.62
Aureobasidium pullulans	0.70	0.27	1.04	0.62
Chaetomium globosum	NT	-	1.60	0.45
Doratomyces microsporus	2.13	1.15	4.54	0.15
Fusarium decemcellulare	1.24	0.38	1.52	0.40
Graphium rigidum	3.86	0.68	4.33	2.91
Oidiodendron griseum	1.13	0.42	1.96	0.29
Paecilomyces varioti	0.42	0.10	2.79	1.45
Penicillium frequentans	0.45	0.06	NT	-
Phialophora mutabilis	2.54	1.31	3.58	1.07
*Polystictus versicolor	NT	-	29.93	5.65
Pycnostysanus sp.	2.24	0.45	NT	-
Pyrenochaeta sp.	2.95	1.32	5.65	1.72
Trichoderma viride	0.66	0.29	0.99	0.16

*White rotting Basidiomycete included for comparison.

Experiment 1 - Blocks measured 20 x 20 x 20mm

Experiment 2 - Blocks measured 30 x 20 x 10mm

Each value was the mean obtained from four replicate blocks.

Incubation was for 16 weeks at 22°C.

Raw data for Table 4 is shown in Appendix 3.

3.1.4 Predominant Fungi in Untreated Eucalyptus obliqua sapwood at Three Time Intervals

The fungi present in untreated *Eucalyptus obliqua* sapwood at 12, 24 and 36 week time intervals after emplacement were studied using stakes positioned at Grove, Tasmania.

Fungi present at all three time intervals were *Trichoderma viride*, *Fusarium* spp., *Oidiodendron griseum* and *Penicillium* spp. and unidentified laccase-positive Basidiomycetes. *Trichoderma viride* appeared to be the dominant organism at 24 weeks exposure, although *Phialophora mutabilis* was prevalent after 36 weeks emplacement. At least three separate Basidiomycete species were isolated after 36 weeks exposure.

Other organisms isolated and identified were *Oidiodendron griseum* (12 weeks emplacement), *Cephalosporium acremonium* (24 and 36 weeks), *Alternaria* sp. (24 weeks), *Mucor* sp. (24 weeks), *Graphium* sp. (24 weeks) and *Candida* spp. (24 weeks).

3.2 Bacteria

3.2.1 Identification of Selected Bacteria Isolated from Tasmanian CCA-Treated Eucalyptus sp. Hardwood Poles

Twenty-six randomly selected bacterial isolates from the ground-line of CCA-treated poles were tested for the ability to degrade a range of substrates found in hardwoods. All identified isolates (Tables 5-6) produced either cellulases or pectic enzymes.

Four isolates characterised were Gram-positive spore-forming rods belonging to three species of the genus *Bacillus* (Table 6). Two Gram-negative yellow-pigmented rods without endospore production were placed in the genus *Flavobacterium*,

Table 5.

Production of wood-degrading enzymes by bacteria isolated from Tasmanian CCA-treated Eucalyptus spp. transmission poles.

Isolate	Bacterium	Cx-cellulase activity	RBBR-cellulose dye release	Xylanase activity	Amylase activity	Pectic enzyme activity		Laccase activity
						pH 2.0	pH 8.8	
N2NA	Bacillus carotarum (Strain 1)	+++	-	-	+	-	-	-
N10NA	Bacillus carotarum (Strain 2)	-	-	-	+	-	+	-
S6SA	Bacillus circulans	+	-	-	+	-	+	-
S9NC	Bacillus megaterium	+	-	+	+	-	+	-
N6NB	Cellulomonas sp.	++	+	-	-	-	-	+
S8N	Cellulomonas sp.	++	+	+	-	-	+	+
S10N	Cellulomonas sp.	+	+	+	+	+	+	-
S2S	Flavobacterium ferrugineum	-	-	-	++	-	+	-
N4NA	Flavobacterium sp.	++	+	-	+++	+	+	-
N6NA	Nocardia rugosa	+	-	-	++	-	-	-
S7NA	Streptomyces sp.	+++	-	-	+	-	+	-

All isolates identified had Cx-cellulase or pectic enzyme activity.

Bacterial Cx-cellulase production was determined using 1ml aliquots of culture filtrate (20d growth at 22°C) added to 10ml of 0.5% NaCMC (pH 5.5) in 0.1M acetate buffer and incubated for 1h at 50°C.

Key: + - 1-20% reduction in viscosity NaCMC
++ - 20-50%
+++ - more than 50%

Release of dye from RBBR-cellulose was assessed after 30d growth at 22°C. All positive isolates produced faint colour only.

Both xylanase and amylase activities were assessed by the agar well diffusion method using 0.1ml aliquots from culture filtrates (10d growth at 22°C).

Amylase activity: + - 1-2mm clearing
++ - 2-6mm
+++ - >6mm

Pectic enzyme production was assessed by polyacrylamide gel electrophoresis. Cultures were grown for 10d at 22° C. Polygalacturonases were the only enzyme form detected, although no assay for pectin lyases was made.

Laccase activity as determined using the spot-test method of Harkin and Obst (1973). Cultures were grown on TYE for 6d at 22°C before testing.

Table 6

Biochemical characteristics of bacterial isolates

Isolate	Morphology	Identification	Gram Stain	Motility	Flagella	Endospore	Catalase	Voges-Proskauer	*Glucose Acid, Gas Production	Mannitol	Anaerobic agar (growth)	Citrate (growth)	Indole	50^oC (growth)
N2NA	Rods (chains) 1.0-1.2-3.0-5.0 µ. White, opaque colony.	*Bacillus carotarum* (strain 1)	(+)	-	-	+	+	-	-	-	-	+	+	+
N10NA	Rods (large) 1.2-1.5-3.5-5.0 µ. White colony.	*Bacillus carotarum* (strain 2)	+	-	-	+	+	-	-	-	-	+	+	-
S6SA	Rods (large, in chains) 1.0-1.5-3.0-5.0 µ. Brown pigment.	*Bacillus circulans*	(-)	+	Perit.	+	+	+	+	+	-	+	-	-
S9NC	Rods (large) 2-3 cell chains. 1.0-1.5-3.0-5.0 µ. Brown pigment.	*Bacillus megaterium*	+	-	-	+	+	-	+	+	-	+	-	(+)
N6NB	Rods 0.8-1.0-1.0-1.5 µ. White, opaque.	*Cellulomonas* sp.	(+)	+	Polar	-	+	+	+	+	+	-	-	-
S8N	Rods 0.5-0.8-0.9-2.0 µ. White, opaque.	*Cellulomonas* sp.	(+)	+	Polar	-	+	+	+	+	+	-	-	+
S10N	Rods, small, slimy. 0.4-0.6-1.0-1.5 µ. White, opaque.	*Cellulomonas* sp.	(+)	+	Polar	-	+	+	+	-	+	-	+	-
S2S	Rods (small 0.6-0.9-12.-1.8 µ. Brown-orange pigment, shiny.	*Flavobacterium ferrugineum*	-	+	Perit.	-	+	+	+	-	-	-	-	-
N4NA	Rods 0.8-1.0-2.0-5.0 µ. Translucent, yellow pigment.	*Flavobacterium* sp.	-	+	Perit.	-	+	+	-	-	-	-	+	-
N6NA	Actinomycete. White, true mycelium.	*Nocardia rugosa*	+	-	-	-	+	-	-	-	-	+	-	(+)
S7NA	Actinomycete. Dark grey, true mycelium produced.	*Streptomyces* sp.	+	-	-	-	+	-	-	-	-	-	-	+

( ) - Variable Gram stain or very slow growth
Perit. - Peritrichous flagella
* - No gas produced by any isolate

Table 6 (continued)

Biochemical characteristics of bacterial isolates

Identification	7% NaCl (growth)	$NO_3^- \rightarrow NO_2^-$	Starch hydrolysis	Pigment: Water soluble	Pigment: Water insoluble	Resistance to lysozyme	Acid-fast	Characteristics used in identification
Bacillus carotarum (strain 1)	+	+	+*	-	-	-	NT	Short chains of cells, G(+)ve, spores produced, aerobic, grew in 7% salt, not resistant to lysozyme, starch weakly hydrolysed → red border.
Bacillus carotarum (strain 2)	+	-	+*	-	-	-	NT	As above.
Bacillus circulans	+	-	+	+	-	+	NT	G variable, spore-forming rod, starch hydrolysed, indole not produced, acid formed from mannitol.
Bacillus megaterium	+	-	+	+	-	-	NT	G(+)ve sporing rod, grew 7% salt, no growth anaerobic agar, not resistant to lysozyme, brown pigment formed on agar media.
Cellulomonas sp.	+	+	-	-	-	-	NT	G variable, non-sporing rod, irregular shaped, attacked cellulose.
Cellulomonas sp.	+	+	-	-	-	+	NT	As above.
Cellulomonas sp.	+	-	+	-	-	+	NT	As above.
Flavobacterium ferrugineum	+	-	+	-	+	-	NT	G(-)ve, orange pigmented rod, spores not formed, acid from glucose, hydrolysed starch, grew in 7% salt.
Flavobacterium sp.	-	-	+	-	+	+	NT	G(-)ve, orange pigmented rod, spores not formed.
Nocardia rugosa	+	(+)	+	(+)	-	+	+	G(+)ve filamentous rods, white, true mycelium produced in 7d culture at 22° C. Mycelium easily fragments after 20h at 22°C.
Streptomyces sp.	+	+	+	-	+	+	NT	G(+)ve, grey, true mycelium produced. Does not fragment easily, oxidative, earthy odour.

( ) - Very low levels of activity or production.
* - red border produced after I_2 staining.
Bacteria were grown on tryptic yeast extract agar (TYE) medium.
NT = not tested.

whilst three strongly cellulolytic Gram-positive asporogenous rods were identified as Cellulomonas spp. None of the three Cellulomonas spp. examined in this investigation corresponded to descriptions of Cellulomonas flavigena, the sole species described in Bergey's Manual of Determinative Bacteriology (1974). Two cellulolytic Actinomycetes were characterised as Nocardia rugosa and Streptomyces sp.

3.2.2 Aspects of the Enzymology of Bacteria Isolated from Tasmanian CCA-Treated Hardwood Poles

Of the 26 bacterial isolates examined, nine produced cellulases as measured by Cx-cellulase (EC 3.2.1.4) activity. Of these nine isolates, one Flavobacterium sp. and three Cellulomonas spp. exhibited dye-release with RBBR-cellulose (Table 5). All isolates that released dye from RBBR-cellulose also had Cx-cellulase activity.

Eight isolates tested showed activity on larch xylan when tested by the agar well diffusion technique. Two Cellulomonas spp. and Bacillus megaterium were identified xylanase producers. Amylase activity was widespread amongst the bacteria examined: 19 isolates or more than 70% of those tested could degrade starch. One Cellulomonas sp. had faint amylase action which is atypical of the genus (Bergey's Manual of Determinative Bacteriology, 1974). Both Flavobacterium ferrugineum and another unidentified Flavobacterium sp. (N4NA) were potent producers of amylases. Two strains of Bacillus carotarum produced a red-pigmented border during starch hydrolysis; a distinctive aid to their identification.

Pectic enyme activity was shown by eight bacterial isolates using polyacrylamide gel electrophoresis. Two levels of pH were used for gel incubation. The high incubation pH (8.8) allowed a higher level of enzyme detection than did pH 2.0. All pectic enzymes detected were polygalacturonases (EC 3.2.1.15). No pectin esterase (EC 3.1.1.11) activity was demonstrated and no assay for pectin lyases (EC 4.2.2.2) was made.

Laccase (EC 1.10.3.2) production was rare as only two bacterial strains were positive from the 26 isolates examined. Both positive bacteria were *Cellulomonas* spp.

3.2.3 Attack of Eucalyptus obliqua Sapwood by Bacterial Isolates

No evidence of bacterial attack on intact wood cell walls was apparent using SEM and light microscopy of 20 µm thin wood sections after 14d exposure at 22C (Plates 12-17). However, both *Nocardia rugosa* and a *Streptomyces* sp. showed growth in some parenchyma cells (Plates 12-14), whilst *Streptomyces* sp. grew vigorously on the surface of sections examined with SEM (Plates 16-17).

3.2.4 Aspects of Bacterial Wood Colonization

Bacterial colonization of untreated *E. obliqua* sapwood stakes emplaced at Grove, Tasmania, was marked. The mean estimated bacteria number was 1.2×10^4/g wood (S.E. 4.2×10^2) at 12 weeks emplacement, declining to a mean of 8.5×10^3/g wood (S.E. = 5.9×10^2) at 24 weeks and 5.3×10^3/g wood (S.E. = 6.6×10^2) after 36 weeks exposure.

Four isolates from a random selection of 12 bacterial strains from the stakes had pronounced cellulolytic activity when grown on NaCMC agar. These isolates were all identified as *Bacillus* spp.

Bacteria appeared to be the dominant flora in creosote-treated *E. obliqua* sapwood stakes inserted for 36 weeks at

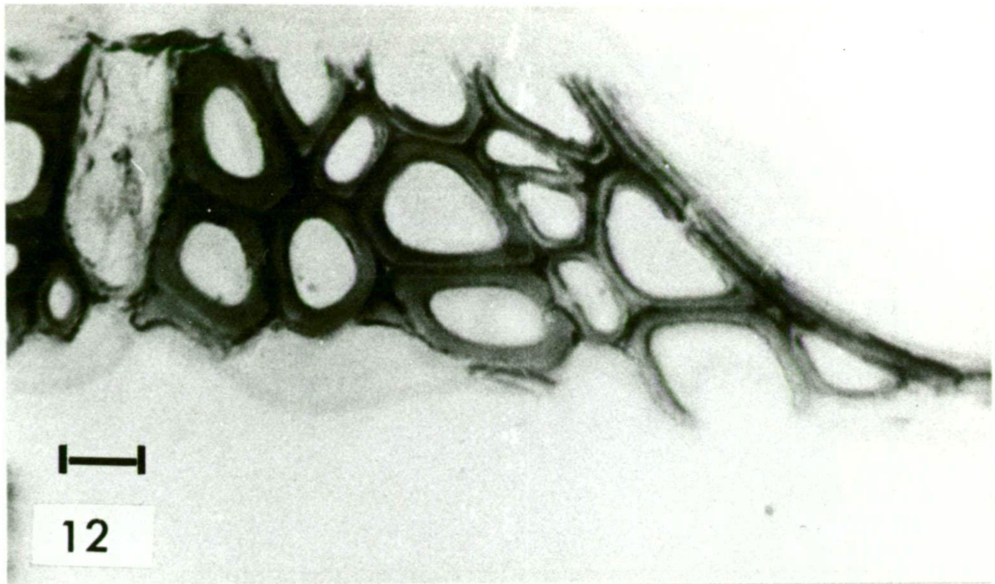
Bacterial attack of *Eucalyptus obliqua* sapwood

Bacteria were grown for 14d at 22^{o} C on wood sections measuring 10mm x 10mm x 20 µm in a water-saturated environment.

Plate 12. Filamentous *Streptomyces* sp. in ray cell. Transverse section (TS). Bar = 10µm.

Plate 13. *Nocardia rugosa* cells in *E. obliqua* paratracheal parenchyma cell (TS). Bar = 5µm.

Plate 14. *Nocardia rugosa* cells attacking previously cut ray and fibre cell walls (TS). Bar = 5µm.



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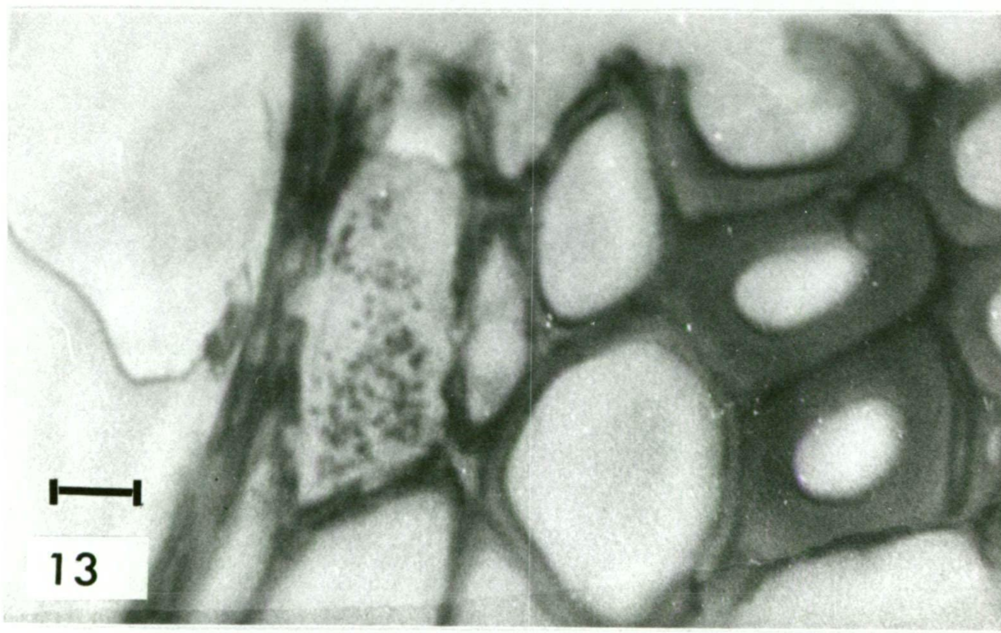

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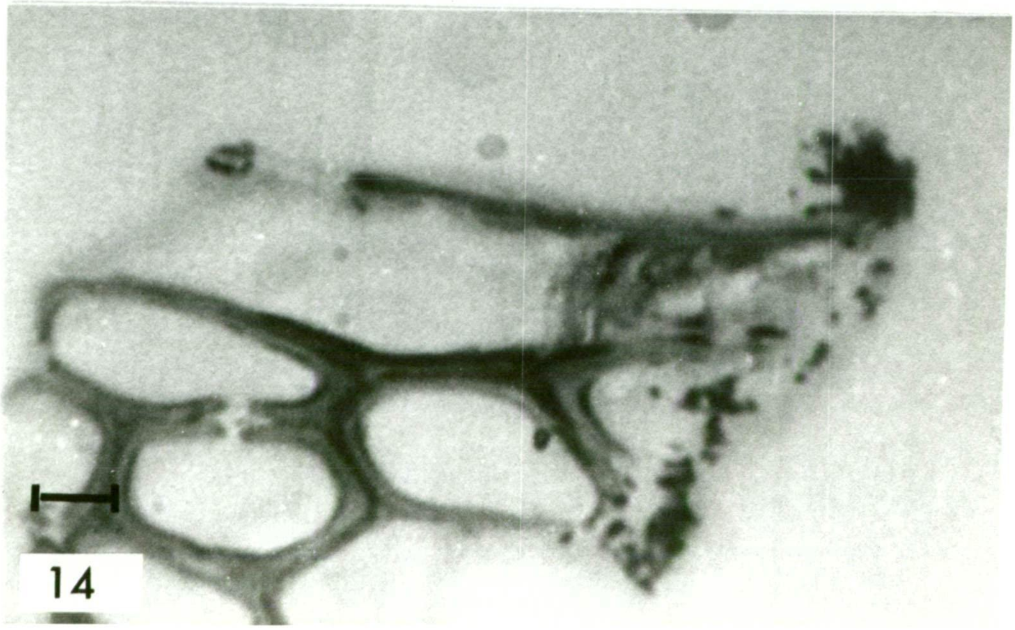

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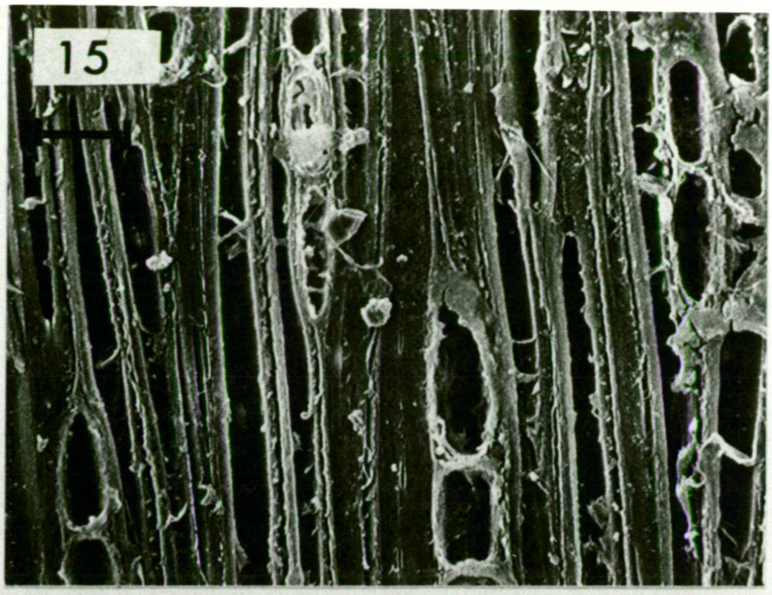
<u>Bacterial attack of Eucalyptus obliqua sapwood (continued).</u>

<u>S.E.M. Studies</u>

Plate 15 Uninoculated control. A tangential longitudinal section (TLS). Note the cut, ovoid medullary ray cells. Bar = 15µm.

Plate 16 <u>Streptomyces</u> sp. on surface of <u>E. obliqua</u> wood, showing chains of aerial spores. Bar = 6 µm.

Plate 17 <u>Streptomyces</u> sp. (TLS). Strands of slime and branching hyphae are visible. Little degradation of the cell wall was evident at this stage of attack. Bar = 6µm.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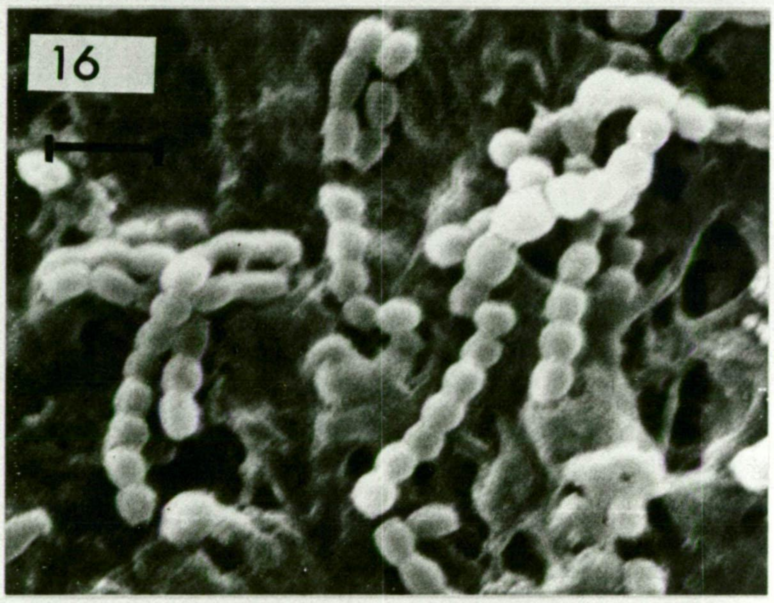
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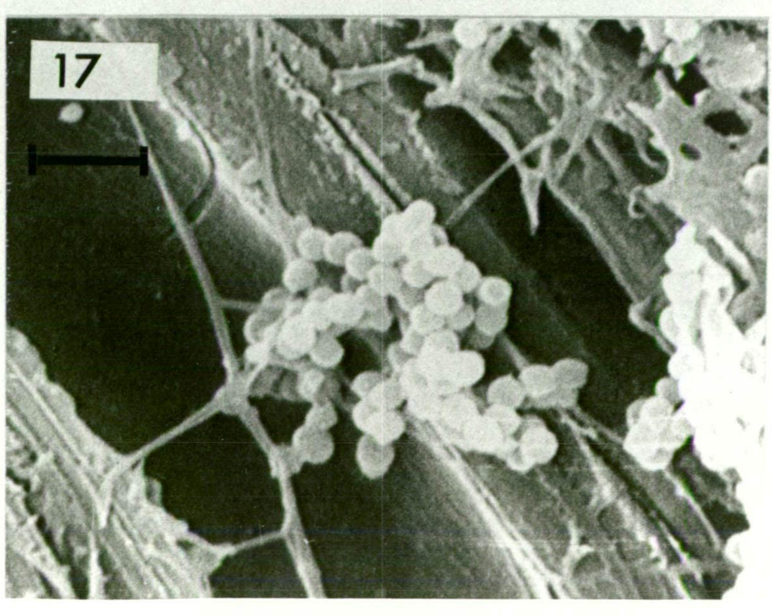
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Grove, Tasmania. Counts ranged from 1.7 x 10^3-4.8 x 10^3 cells per gram sawdust. No Cx-cellulase activity could be demonstrated for any of seven selected strains when tested in culture. Counts ranging between 2.3 x 10^3-5.4 x 10^3 cells per gram sawdust were made from NaPCP-treated woods emplaced at Grove for 36 weeks. Estimated bacterial colony numbers in stakes treated with the remaining preservatives were not greater than 2 x 10^3 cells/g wood, and were as a result, probably insignificant.

Creosote-treated Eucalyptus maculata pole stubs positioned at Coff's Harbour, N.S.W., for three years, produced estimated bacterial numbers of 1.7 x 10^3-1.8 x 10^4 cells/g wood. Copper-chrome-arsenic-treated Pinus radiata stubs had estimated numbers of bacteria ranging from 3.1 x 10^3-7.9 x 10^3 cells/g wood. There was no evidence of extensive bacteria colonization in any treated pole stub emplaced at Warrane, Tasmania.

3.3 Relationship between Fungal and Bacterial Propagule Counts in Sawdust Samples from Tasmanian CCA-Treated Hardwood Poles

Positive regressions were observed between fungal propagule estimates and bacterial colony counts in two sets of wood samples examined (Fig. 2).

Determination of r^2, being a measure of the total variability in one variate which can be accounted for in the other (Steel and Torrie, 1960) showed the relationship between fungal propagule and bacterial counts (in both samples) to be relatively weak. The unexplained variability for Sample A [1 - r^2 x 100%] was 60% and for Sample B, 70%.

Figure 2

Relationship between bacterial and fungal propagule numbers in sawdust samples from CCA-treated *Eucalyptus* sp. poles

Bacterial colony numbers were counted on 0.3% tryptic soy agar plus 0.1% yeast extract (TYE), 0.01g sawdust per plate. The incubation period before counting was 4d at 22 C. Fungal propagule numbers were enumerated on 0.25% swollen cellulose agar, 0.01g sawdust per plate. The incubation period was 12d at 22 C.

○ Sample A – poles situated in Northern Tasmania.
$b = 0.55$, $r = 0.63$ ($r^2 = 0.40$).

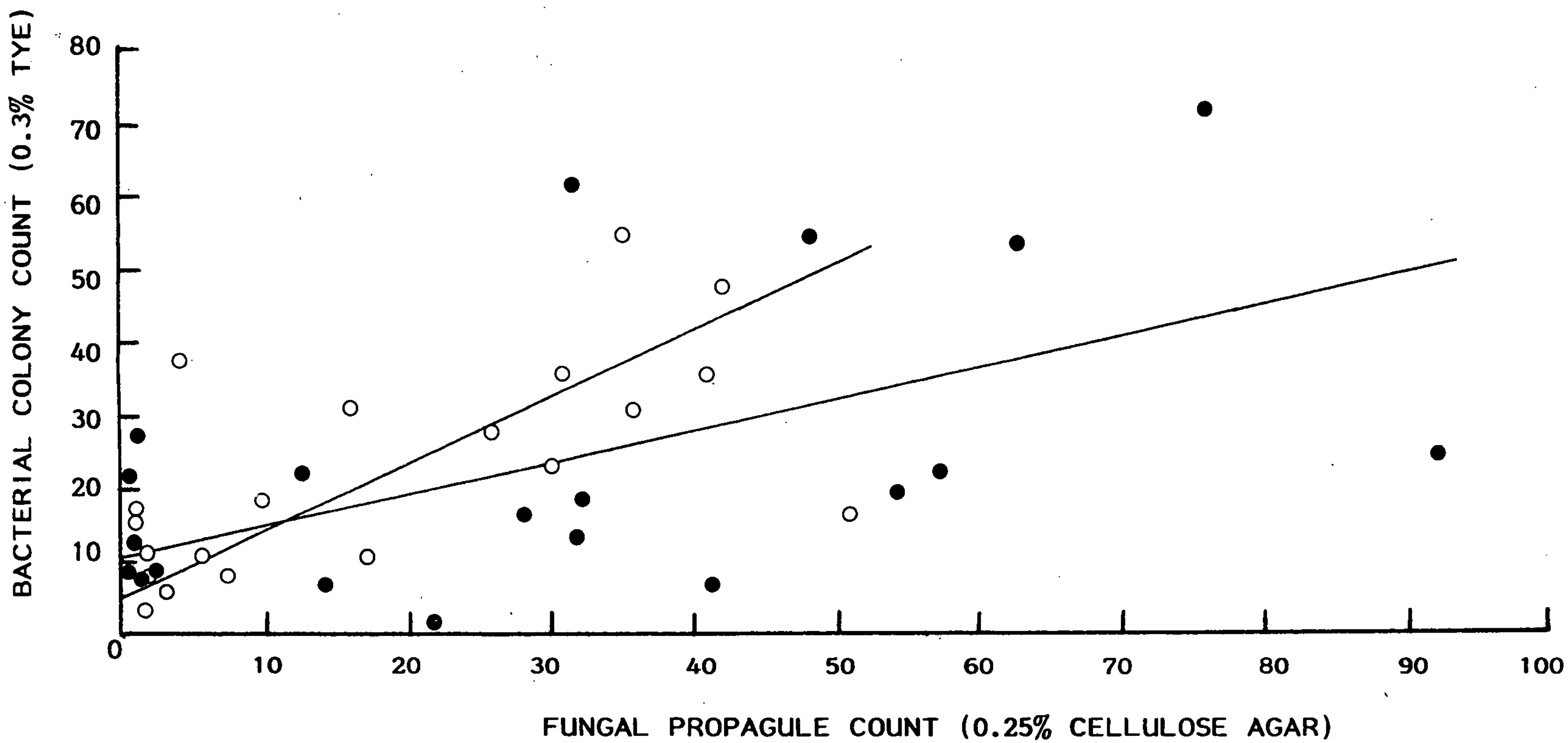
● Sample B – poles situated in Southern Tasmania.
$b = 0.39$, $r = 0.54$ ($r^2 = 0.29$).

b = regression coefficient

r = correlation coefficient

The predominant fungi isolated from Sample A woods were *Phialophora mutabilis*, *Ambylosporium* sp., *Penicillium* spp., and various yeasts; whilst the main fungal species in Sample B woods were *Phialophora mutabilis*, *Penicillium* spp., *Trichoderma viride* and *Fusarium* spp.

The highest estimated bacterial population in this examination was 7.3×10^3 cells/g CCA-treated wood.


BACTERIAL COLONY COUNT (0.3% TYE)
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
FUNGAL PROPAGULE COUNT (0.25% CELLULOSE AGAR)

3.4 Development of Assay Techniques for the Comparative Assay of Wood Degradation

Improved methods of wood degradation assessment were considered necessary to increase the accuracy of preservative efficacy testing and to shorten the time needed to evaluate preservatives in the field. Two new techniques developed in this study were the wood Cx-cellulase assay and a chitin assay for the estimation of mycelial biomass in wood.

3.4.1 Development of the Cx-cellulase Assay

Several parameters of the Cx-cellulase assay were investigated to establish optimal conditions for the technique.

The optimal pH for Cx-cellulase activity in the sawdusted samples from soft-rotted power transmission poles was 5.5 (Fig. 3). Differences in relative activities at pH extremes may be attributable to the different fungal floras present in the three samples.

The optimal temperature of incubation of the sawdusted wood samples was between 45-55 C (Fig. 4). A sharp decline in activity was noted at temperatures higher than 55-65 C, presumably due to denaturation of the enzymes in the sawdust.

The Cx-cellulase activity was markedly inhibited at 0 C (Fig. 5), thus enabling assays of wood samples to be made in batches, rather than individually. Samples could then be placed in ice following incubation to await viscometry. Allowance for the low level of enzyme activity at 0 C was possible using the standard curve shown (Fig. 5).

Concentrations of NaCMC between 0.1% and 1.0% were suitable for use in viscometric determinations. A 0.4% or 0.5% NaCMC concentration was found to be convenient in this study.

Figure 3

Effect of pH on carboxymethyl cellulase activity of three wood samples

Sawdust samples (0.3g) were incubated with 10ml 0.4% NaCMC in citrate-phosphate buffer of varying pH for 30 min at 30 C. The sawdust samples were obtained from two failed *Eucalyptus obliqua* poles (Poles 1 and 2) and one *E. amygdalina* post (Pole 3). Each determination was the mean of duplicate samples.

○ = Pole 1;

● = Pole 2;

□ = Pole 3.

Predominant fungi isolated were:

Pole 1 - *Penicillium* spp., *Paecilomyces varioti*, *Phialophora mutabilis*

Pole 2 - *Phialophora mutabilis*, *Penicillium* spp.

Pole 3 - *Penicillium* spp., *Fusarium* spp., *Paecilomyces varioti*

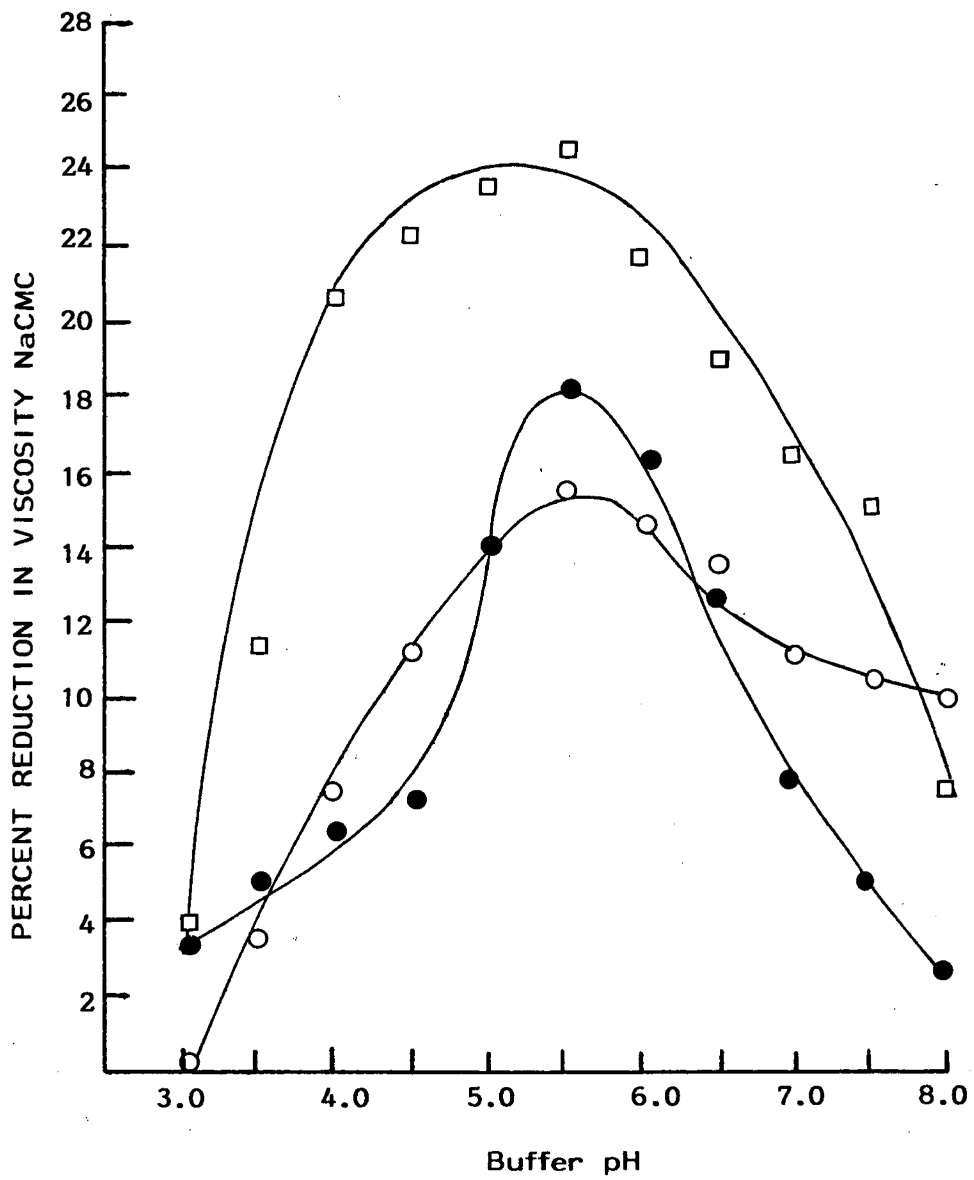

PERCENT REDUCTION IN VISCOSITY NaCMC
28
26
24
22
20
18
16
14
12
10
8
6
4
2
3.0
4.0
5.0
6.0
7.0
8.0
Buffer pH

Figure 4

Effect of temperature on carboxymethyl cellulase activity of three wood samples

Sawdust samples (0.3g) were incubated with 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 45 min at the indicated temperatures. The three wood samples were failed *E. obliqua* poles. Each determination was the mean of duplicate samples.

○ = Pole 1

● = Pole 2

□ = Pole 4.

Phialophora mutabilis, *Penicillium* spp., *Cladosporium* sp., and *Amblyosporium* sp. were the predominant fungi isolated from Pole 4.

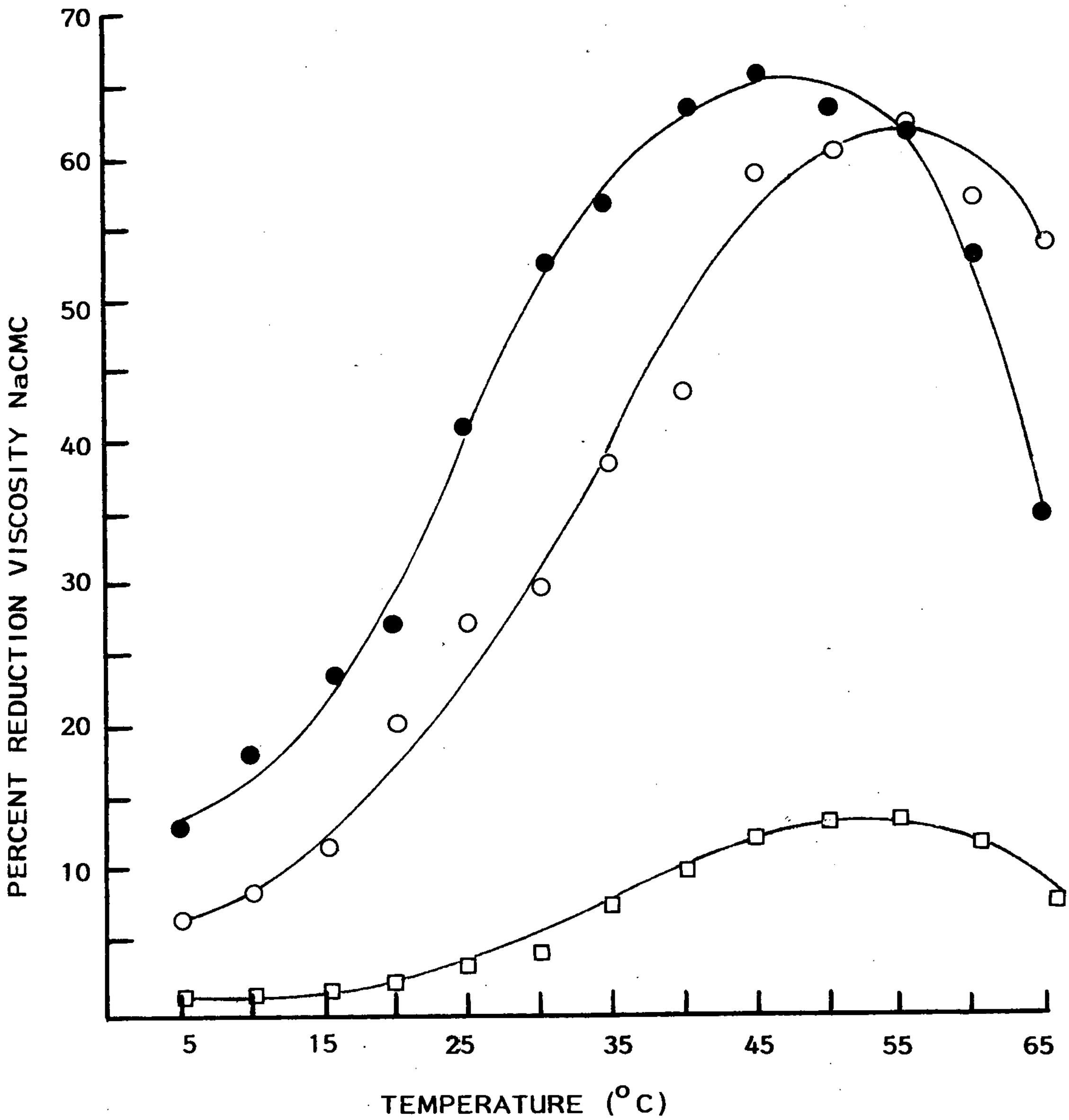
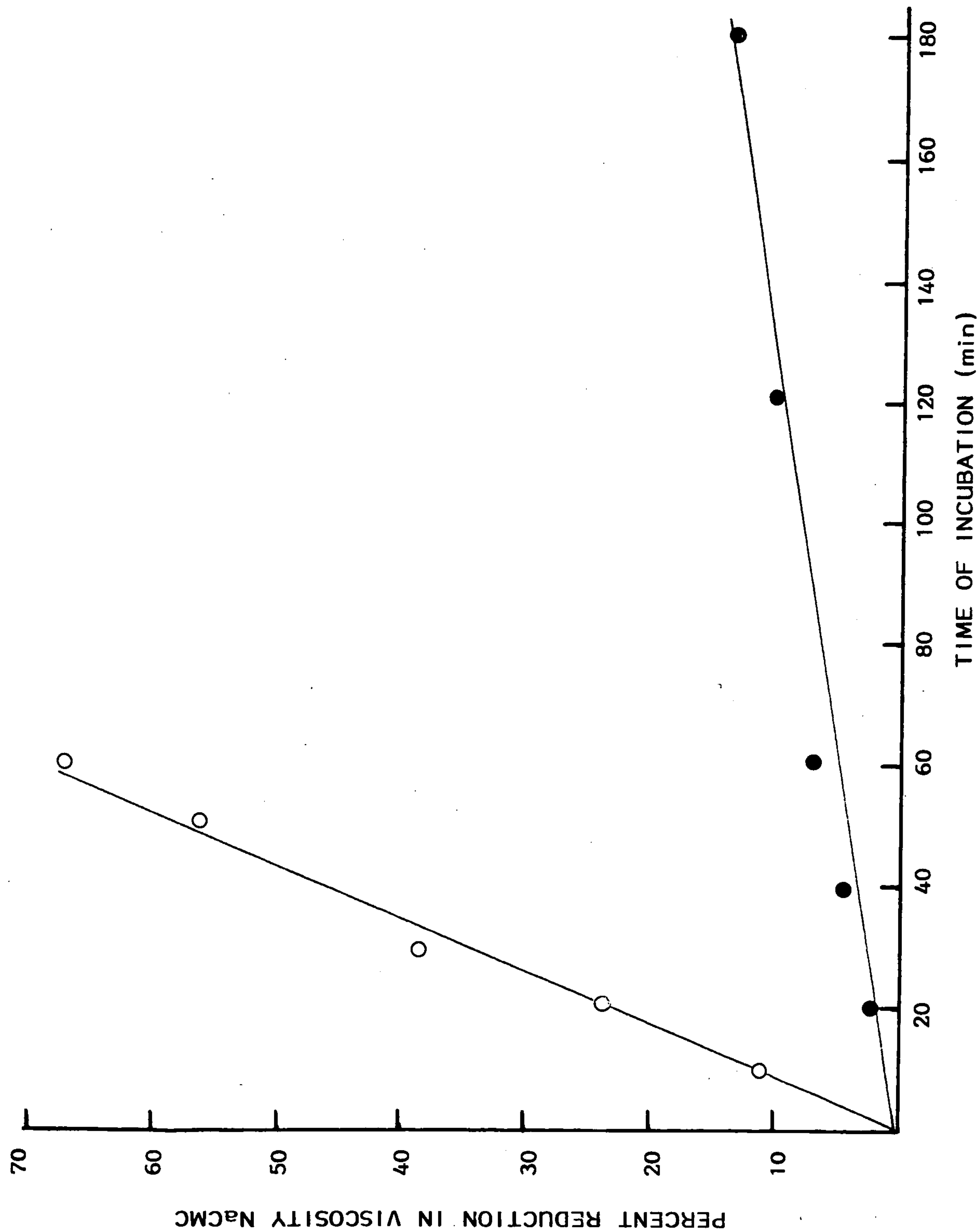

70
60
50
40
30
20
10
PERCENT REDUCTION VISCOSITY NaCMC
5
15
25
35
45
55
65
TEMPERATURE (°C)

Figure 5

Inhibition of carboxymethyl cellulase activity by storage of samples at $0^{o}C$

Pooled *Eucalyptus obliqua* sawdust samples (0.3g) were incubated with 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) at $45^{o}C$ (O), or $0^{o}C$ (●) in ice. Each determination was the mean obtained from duplicate samples.


PERCENT REDUCTION IN VISCOSITY NaCMC
70
60
50
40
30
20
10
TIME OF INCUBATION (min)
20
40
60
80
100
120
140
160
180

3.4.2 Development of a Chitin Assay (for the estimation of fungal biomass in hardwoods)

A method of estimating fungal biomass in wood was desired to complement the Cx-cellulase assay technique. A chitin assay was considered to be an appropriate method.

Application of a slightly modifed method of Ride and Drysdale (1972) to soft-rot infected poles revealed a decreasing chitin content from the pole perimeter to the heartwood (Fig. 6). However, excessively high levels were recorded in the sound heartwood, raising the possibility that phenolic compounds may have interfered with the colorimetric estimation of fungal chitin. Attempts were made to remove such compounds by passing wood digests through columns packed with alumina, polyvinyl polypyrrolidone or (1:4) activated charcoal. None was successful.

An alternative assay was devised, comprising three basic steps:

(i) acid hydrolysis of fungal chitin-containing wood samples, releasing glucosamine amino-sugar, and dilution of the hydrolysates.

(ii) Ion-exchange chromatography.

(iii) Estimation of glucosamine concentration by the colorimetric method of Tsuji, Kinoshita and Hoshino (1969), using MBTH.

To establish optimal conditions for the assay, several parameters were investigated. The standard curve of glucosamine plus MBTH is shown in Appendix 4. For the assessment of the optimal time of acid hydrolysis, glucosamine samples were hydrolysed for various periods at several temperatures. Maximal recovery of glucosamine was achieved after 20-24h hydrolysis at 80 C (Fig. 7).

Figure 6

Assay of the fungal chitin content in wood using an alkaline deacetylation procedure

Wood samples were taken along a radial transect of one soft-rotted *Eucalyptus* sp. wood pole at the ground-line. Sieved sawdust samples (0.2g) were incubated in 5ml of 120% KOH for 1h at 130°C. The chitin contents of the wood samples were estimated using the basic methodology of Ride and Drysdale (1972). Assays were not duplicated.

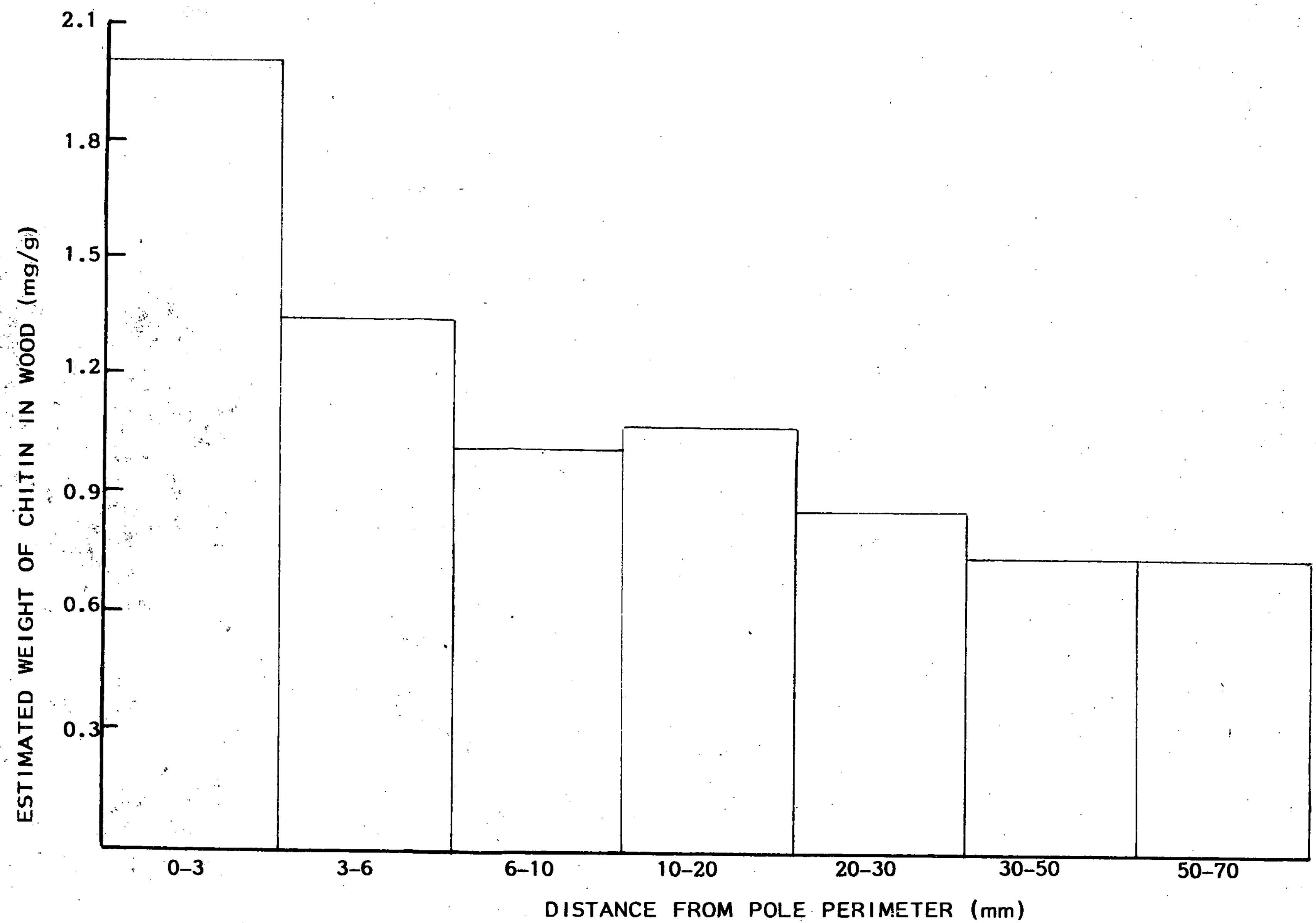
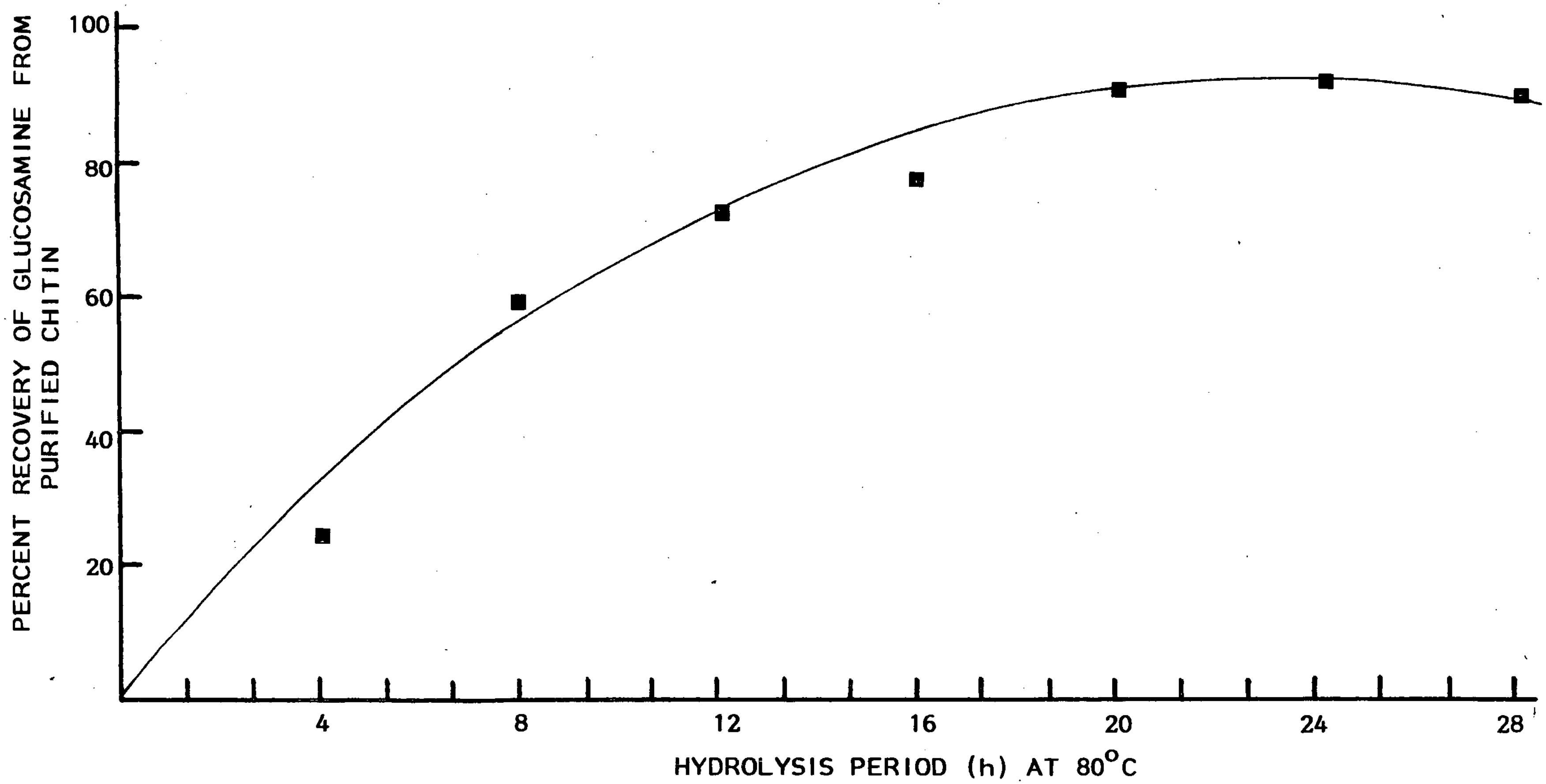

2.1
1.8
1.5
1.2
0.9
0.6
0.3
ESTIMATED WEIGHT OF CHITIN IN WOOD (mg/g)
0-3
3-6
6-10
10-20
20-30
30-50
50-70
DISTANCE FROM POLE PERIMETER (mm)

Figure 7

Hydrolysis time required for maximum % recovery of glucosamine from chitin

Samples of purified chitin (0.02g) were hydrolysed in 5ml of 5N HCl for varying lengths of time prior to filtration and assay. Each value was the mean of duplicate determinations.


PERCENT RECOVERY OF GLUCOSAMINE FROM PURIFIED CHITIN
100
80
60
40
20
4
8
12
16
20
24
28
HYDROLYSIS PERIOD (h) AT 80^{o}C

Amino-acids present in hydrolysed plant tissues would be held and co-eluted with glucosamine from the ion-exchange columns. Therefore it was important to determine whether these compounds had any significant effect on the assay. As detailed in Table 7, L-tryptophan and to a lesser extent L-methionine, L-phenyl alanine and L-ornithine did display some chromogenicity with MBTH reagent.

The reaction products of MBTH with glucosamine showed maximum visible light absorption at 575-675mμ (Fig. 8). This compared well with the corresponding spectra of the reaction products of MBTH and hydrolysed soft-rotted CCA-treated hardwood (Fig. 9). The CCA compounds in the wood showed little apparent interference in the region of maximum absorption, whilst the absorption at lower wavelengths may be due to wood phenolic compounds in the hydrolysate. Little destructive effect on chitin caused by the presence of CCA salts was evident as indicated by the recovery of purified chitin added to CCA-treated and untreated wood samples before hydrolysis (Table 8).

Assay of freshly cut, untreated <u>Eucalyptus obliqua</u> wood samples produced a low but measurable reaction after hydrolysis and ion-exchange chromatography with MBTH reagent (Fig. 10). However, this was of small significance when compared with the reaction produced by soft-rotted CCA-treated <u>E. obliqua</u> sapwood.

3.4.2.1 <u>Assay of the Chitin Levels of Selected Soft-Rot Fungi Cultivated in Liquid Medium</u>

The conversion factor or factors between chitin contents and fungal mycelial dry weights needed to be determined

Table 7

Chromogenic effect, on molar comparative basis, of selected amino acids and MBTH reagent. [Glucosamine is given a relative level of 100%.]

Amino-Acid	% Chromogenicity
L-tryptophan	21.1
L-methionine	6.8
L-phenyl alanine	5.4
L-ornithine-HCl	4.3
L-histidine	3.5
L-asparagine-H_2O	3.0
L-threonine	2.7
L-serine	2.4
L-arginine	1.8
L-tyrosine*	1.8
L-leucine	1.3
L-proline	1.2
L-alanine	0.9
L-glycine	0.1
D,L-valine	0.0
Distilled H_2O	0.0
D-glucosamine-HCl	100

*Largely insoluble.

Figure 8

Visible light absorption spectrum of the reaction products of glucosamine and MBTH reagent.

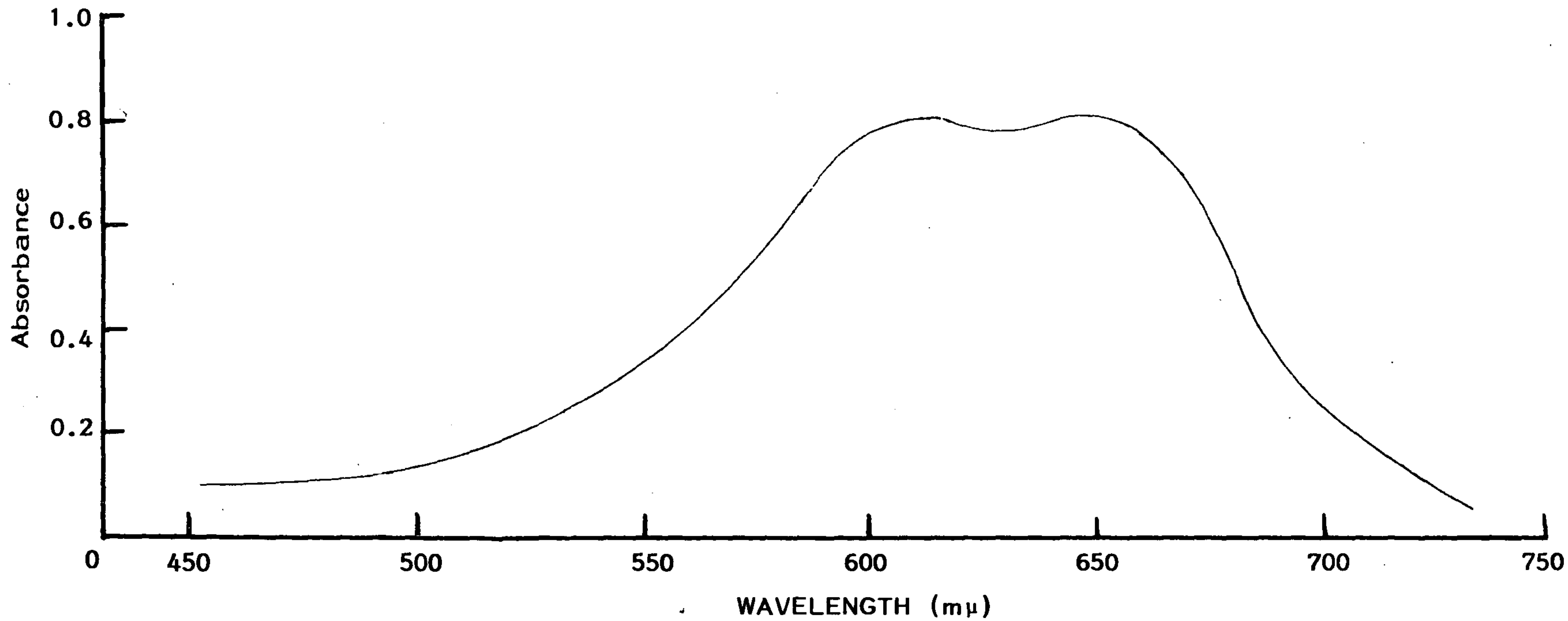

1.0
0.8
0.6
0.4
0.2
Absorbance
0
450
500
550
600
650
700
750
WAVELENGTH (mμ)

Figure 9

Visible light absorption spectrum of the reaction products of hydrolysed CCA-treated soft-rotted wood + MBTH reagent.

The sample was *Eucalyptus obliqua* sapwood (0.2g), hydrolysed in 5ml of 5N HCl for 16h at 80°C.

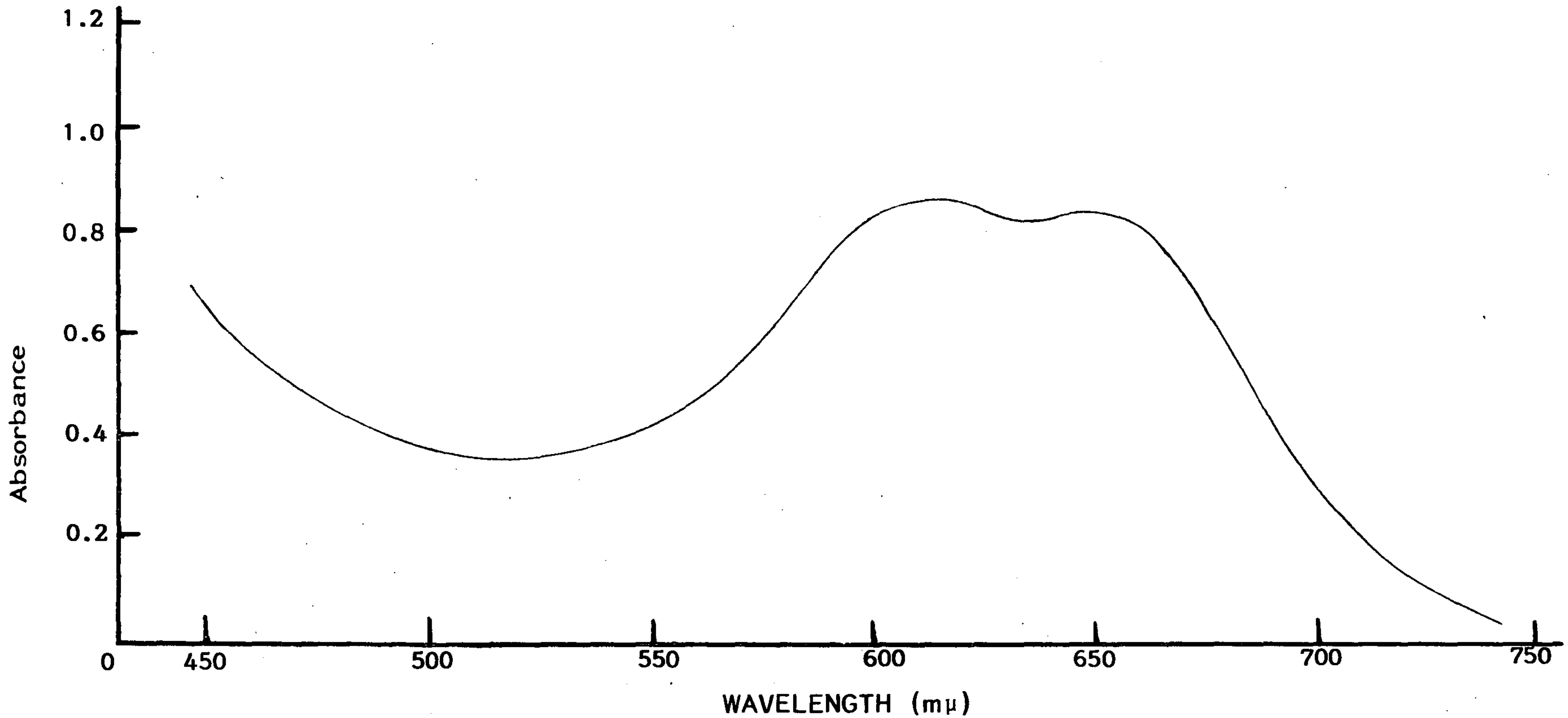

Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
450
500
550
600
650
700
750
WAVELENGTH (mμ)

Table 8

Quantitative recovery of glucosamine from chitin as a percentage of that originally added to sound CCA-treated wood samples prior to hydrolysis.

Sample	Estimated % recovery glucosamine
Sound CCA-treated wood	
Pole 1	78.3
Pole 2	77.8
Pole 3	73.8
Untreated wood	77.5
Purified chitin control	82.5

Purified chitin (0.03g) was added to 0.2g sieved sawdust and hydrolysed in 5ml of 5N HCl for 20h at 80°C. Each value was the mean of duplicate determinations.

Figure 10

Percentage chitin in freshly cut, sound and soft-rotted hardwoods.

The percentage chitin was estimated by comparative colour produced when MBTH was added to hydrolysed wood samples.

- ☐ Freshly cut, untreated _E. obliqua_ sapwood
- ▩ Sound, CCA-treated _E. obliqua_ sapwood
- ⚃ Soft-rotted, CCA-treated _E. obliqua_ sapwood

Sawdusted samples (0.2g) were hydrolysed in 5ml of 5N HCl for 20h at 80 ^{o}C. Each value was the mean of duplicate determinations.

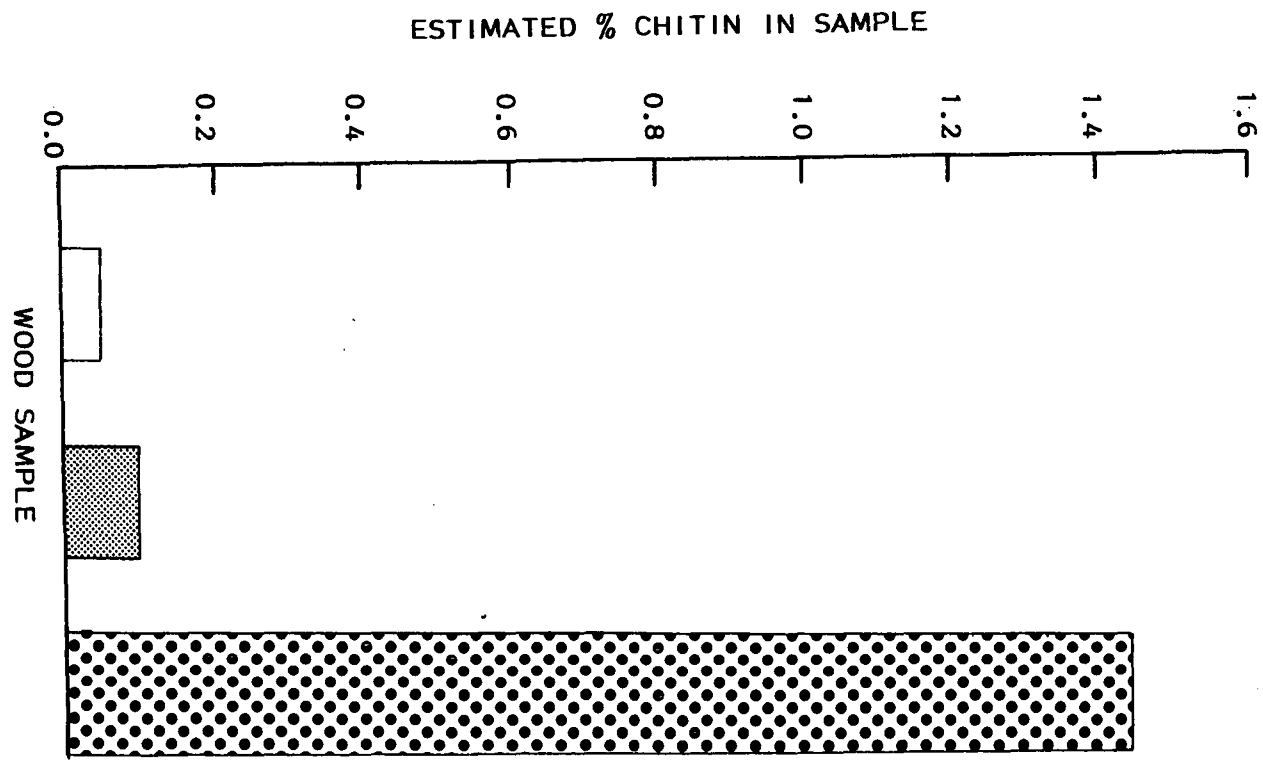
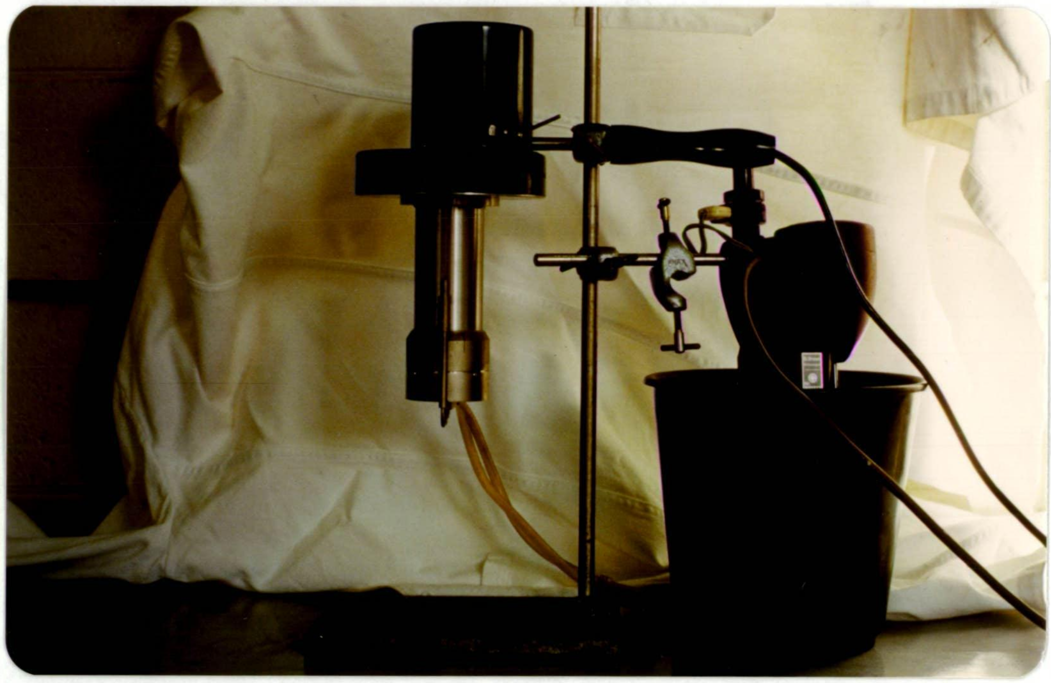

ESTIMATED % CHITIN IN SAMPLE
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
WOOD SAMPLE

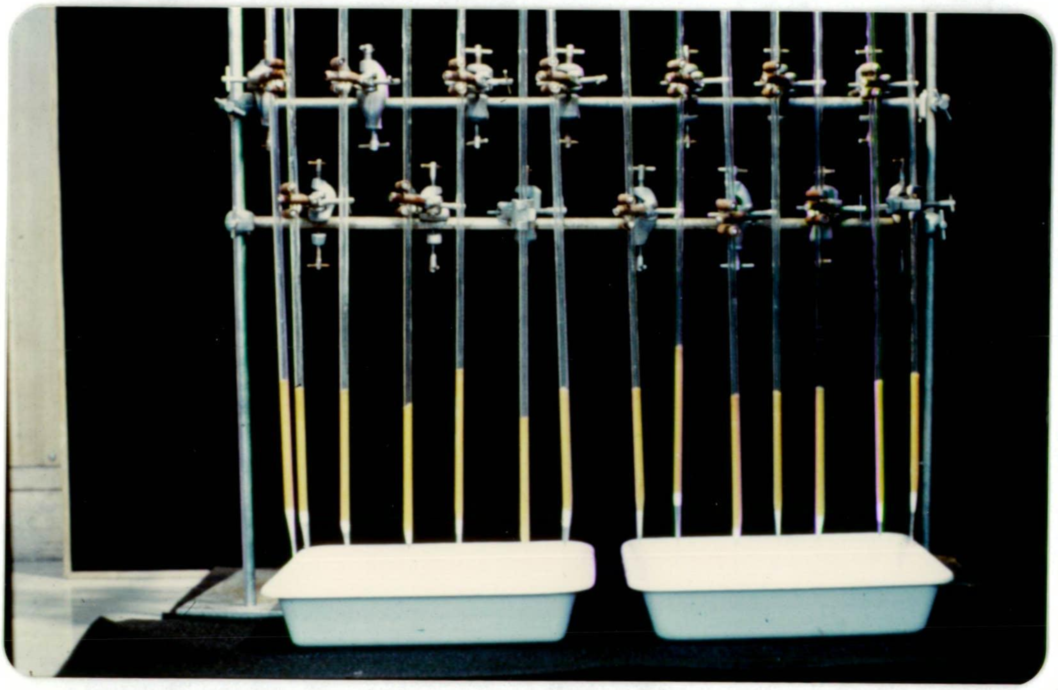
Plate 18 (top)

The Wells-Brookfield microviscometer (with water bath) for Cx-cellulase assays.

Plate 19 (lower)

Glass columns containing Dowex-50W strongly-acid cation exchange resin for the chitin assay (acid hydrolysis technique).





for the calculation of fungal biomass in wood samples. A low-nitrogen nutrient medium was devised in an attempt to approximate the conditions in sapwood.

Trichoderma viride, a relatively fast-growing fungus in synthetic media, had a higher chitin content in the time span studied than Phialophora mutabilis, Oidiodendron griseum and Pyrenochaeta sp. [Figs. 11(i), (ii)]. The chitin levels in the test fungi showed considerable variation over a 30d incubation period, ranging from 2-5% of dry weight in Phialophora mutabilis and Oidiodendron griseum to 8-12% in Trichoderma viride [Fig. 11(ii)]. The total chitin levels in the fungi varied, as expected, with dry weight. However, the chitin content as a percentage dry weight of fungal mycelium also altered depending on the age of the culture in the synthetic medium. Of the four fungi examined, only Phialophora mutabilis had a constant chitin content (w/w) in the time studied [Fig. 11(ii)].

In addition, the chitin levels of 15 soft-rotting fungi were determined following incubation in low-nitrogen liquid medium for 30d at 22 C (Table 9). Chitin contents ranged from 18.4% of dry weight in Doratomyces microsporus to 2.9% in Paecilomyces varioti. An approximate mean of 10% chitin content was obtained for a mixed flora typically found in the poles studied.

3.5 Evaluation of the Bioassay Techniques for the Estimation of Soft-Rot Decay in Eucalyptus sp. Hardwoods

A comparison of fungal propagule numbers obtained on three agar media following plating and incubation of soft-rotted sawdust samples from three CCA-treated wood poles

Plate 20

Release of dye from RBBR-cellulose by fungal isolates (i). From left to right the test tubes contain: RBBR-cellulose (uninoculated), RBBR-cellulose plus *Oidiodendron griseum*; RBBR - cellulose plus *Chaetomium globosum*. The tubes were incubated for 10d at 22°C.

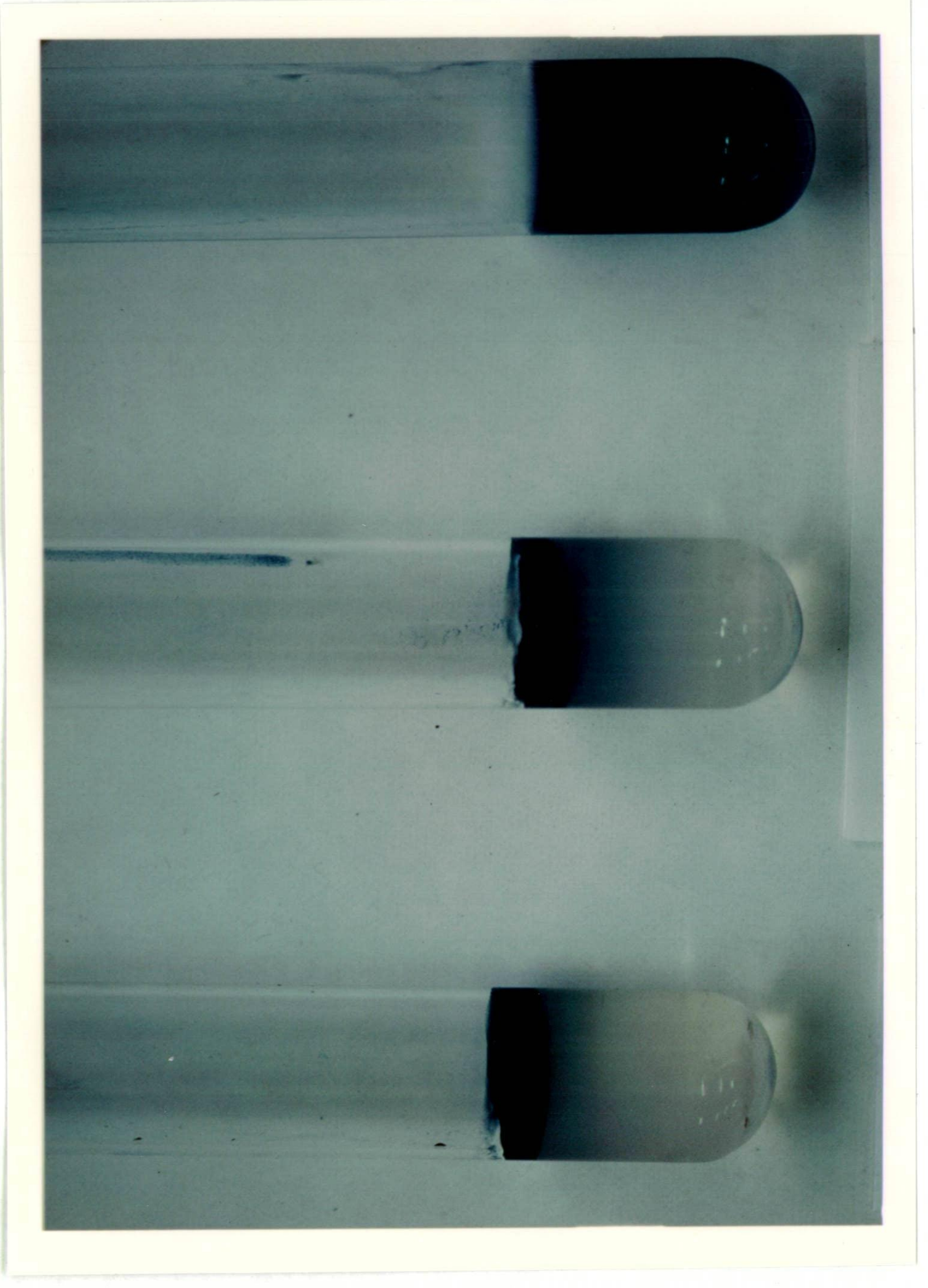


Plate 21

Release of dye from RBBR-cellulose by fungal isolates (ii). From left to right the test tubes contain: RBBR-cellulose (uninoculated), RBBR-cellulose plus *Phialophora mutabilis* (Strain A), RBBR-cellulose plus *Fusarium decemcellulare*. The tubes were incubated for 10d at 22°C.

Figure 11

Chitin content of selected fungi. (i) Variation in estimated fungal chitin content with time.

Fungi were grown in 40ml of low-nitrogen medium at 22°C for 10, 20 or 30d. After harvesting and oven-drying at 100°C for 24h, the fungal mycelia were weighed and hydrolysed in 5ml of 5N HCl at 80°C for 20h. Each value was the mean of duplicate determinations.

Fungal isolates:

○ *Phialophora mutabilis*

● *Oidiodendron griseum*

■ *Trichoderma viride*

▽ *Pyrenochaeta* sp.

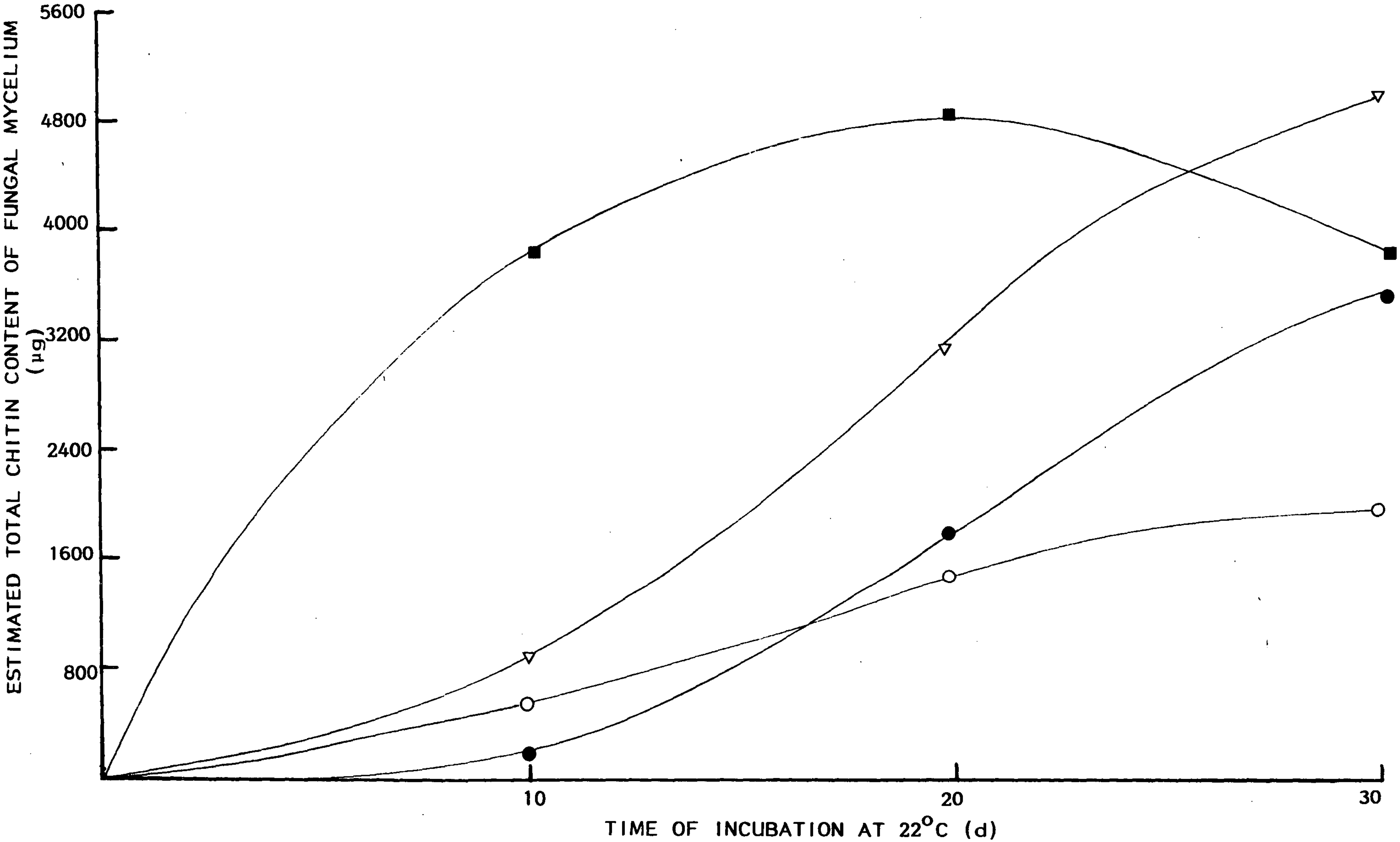

ESTIMATED TOTAL CHITIN CONTENT OF FUNGAL MYCELIUM (µg)
5600
4800
4000
3200
2400
1600
800
10
20
30
TIME OF INCUBATION AT 22°C (d)

Figure 11 (continued)

Chitin content of selected fungi. (ii) Variation in % chitin (w/w) with time.

Fungi were grown in 40ml of low-nitrogen medium at 22 °C for 10, 20 or 30d. After harvesting and oven-drying at 100° C for 24h, the fungal mycelia were weighed and hydrolysed in 5ml 5N HCl at 80 °C for 20h. Each value was the mean of duplicate determinations.

Fungal isolates:

- ○ *Phialophora mutabilis*
- ● *Oidiodendron griseum*
- ■ *Trichoderma viride*
- ▽ *Pyrenochaeta* sp.

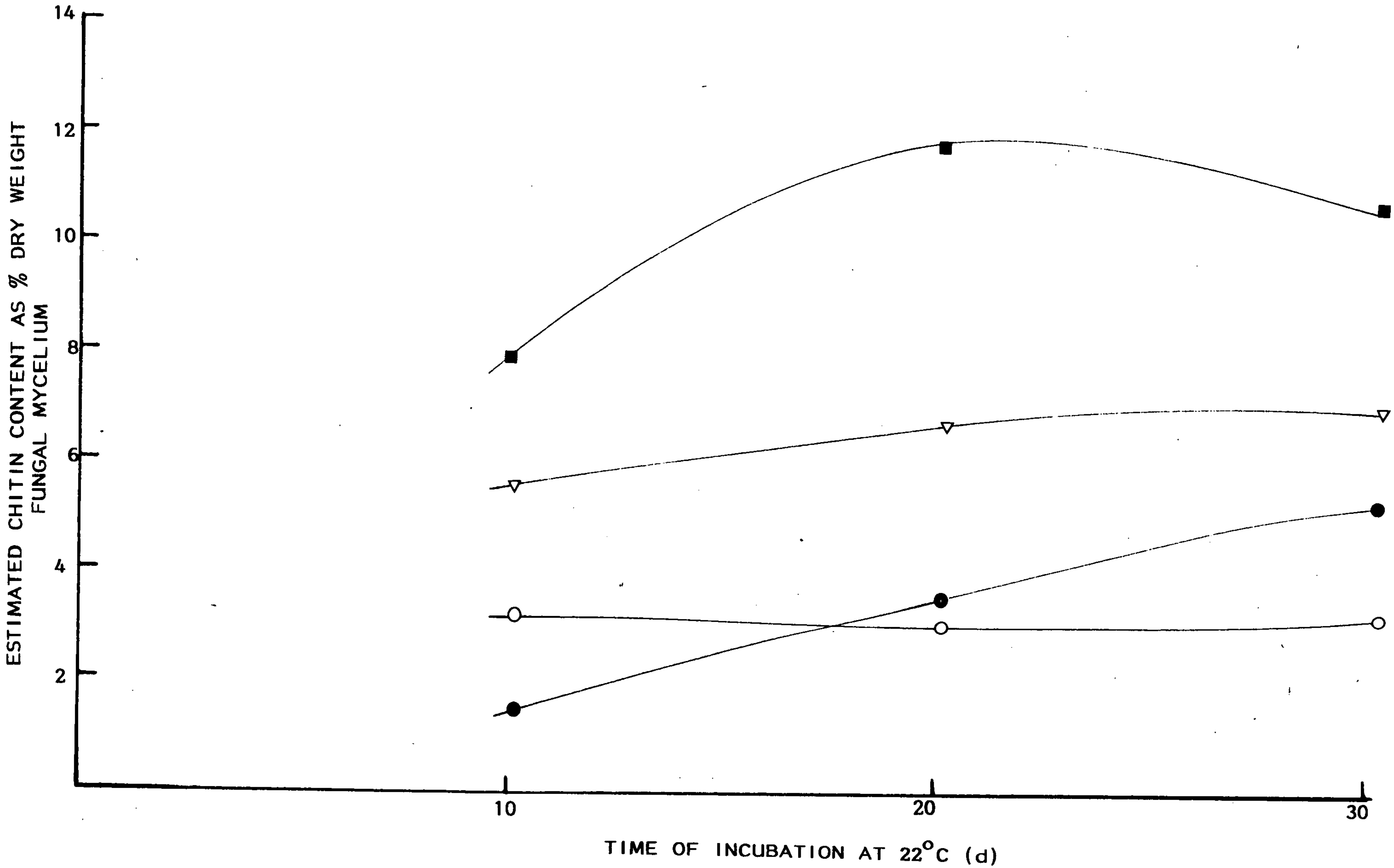

ESTIMATED CHITIN CONTENT AS % DRY WEIGHT FUNGAL MYCELIUM
14
12
10
8
6
4
2
10
20
30
TIME OF INCUBATION AT 22°C (d)

Table 9

Chitin contents of mycelia of selected fungi isolated from soft-rotted Tasmanian Eucalyptus sp. hardwood poles.

Fungal Species	Percent chitin (w/w) of fungal mycelium	
	$\bar{x}$	S.E.
Aspergillus fumigatus Fres.	5.7	0.6
Aureobasidium pullulans (de Bary) Arnaud	16.7	3.1
Cephalosporium acremonium Corda	5.2	1.4
Doratomyces microsporus (Sacc.) Morton and G. Smith	18.4	1.7
Fusarium decemcellulare Brick	7.6	1.4
Graphium rigidum (Pers.) Sacc.	3.0	*
Mucor genevensis Lendner	8.8	0.3
Oidiodendron griseum Robak	8.6	0.7
Paecilomyces varioti Bainier	2.9	0.3
Penicillium frequentans Westling	5.2	1.5
Phialophora mutabilis (Beyma) Schol-Scwarz	4.3	1.2
Pycnostanus sp.	5.5	0.2
Pyrenochaeta sp.	7.7	0.2
Trichoderma viride Pers. ex. Fr.	13.5	3.1
Ulocladium alternariae (Cke.) Simmons	10.6	2.6

*1 sample only.

The fungi were grown in 40ml of low-nitrogen medium for 30d at 22°C. After harvesting, oven-drying and weighing, mycelia were hydrolysed in 5ml of 5N HCl for 20h at 80°C. Each value was the mean of duplicate determinations.

is shown in Figure 12. As expected, a decrease in colony count was observed with increasing selectivity of the agar media, but no major reduction in species diversity was noted. Cellulose agar (0.25% swollen cellulose) was regarded as a suitable medium for the enumeration of wood-degrading fungi from sawdusted samples.

The results of a comparison between the fungal propagule count, the Cx-cellulase assay, the chitin assay and microscopic estimation of cavity formation using three soft-rotted E. obliqua hardwood poles is detailed in Figure 13. A good correlation between the techniques was evident, with all methods indicating decreasing fungal activity with increasing radial distance from the pole perimeter; a characteristic of soft-rot attack. A closer agreement existed between the chitin assay and microscopic estimates, than between the chitin assay, and the Cx-cellulase assay and the fungal propagule count.

Estimates of fungal biomass in wood using the chitin assay developed in this study were two-three times more sensitive than the chitosan (alkaline deacetylation) technique used by Ride and Drysdale (1972) (Figure 6).

3.6 The estimation of Soft-Rot Degradation of Eucalyptus sp. Hardwoods in the Field: Assessment of Assay Techniques

Four field trials of wood preservatives were utilized to appraise the various techniques for the estimation of wood degradation.

Figure 12

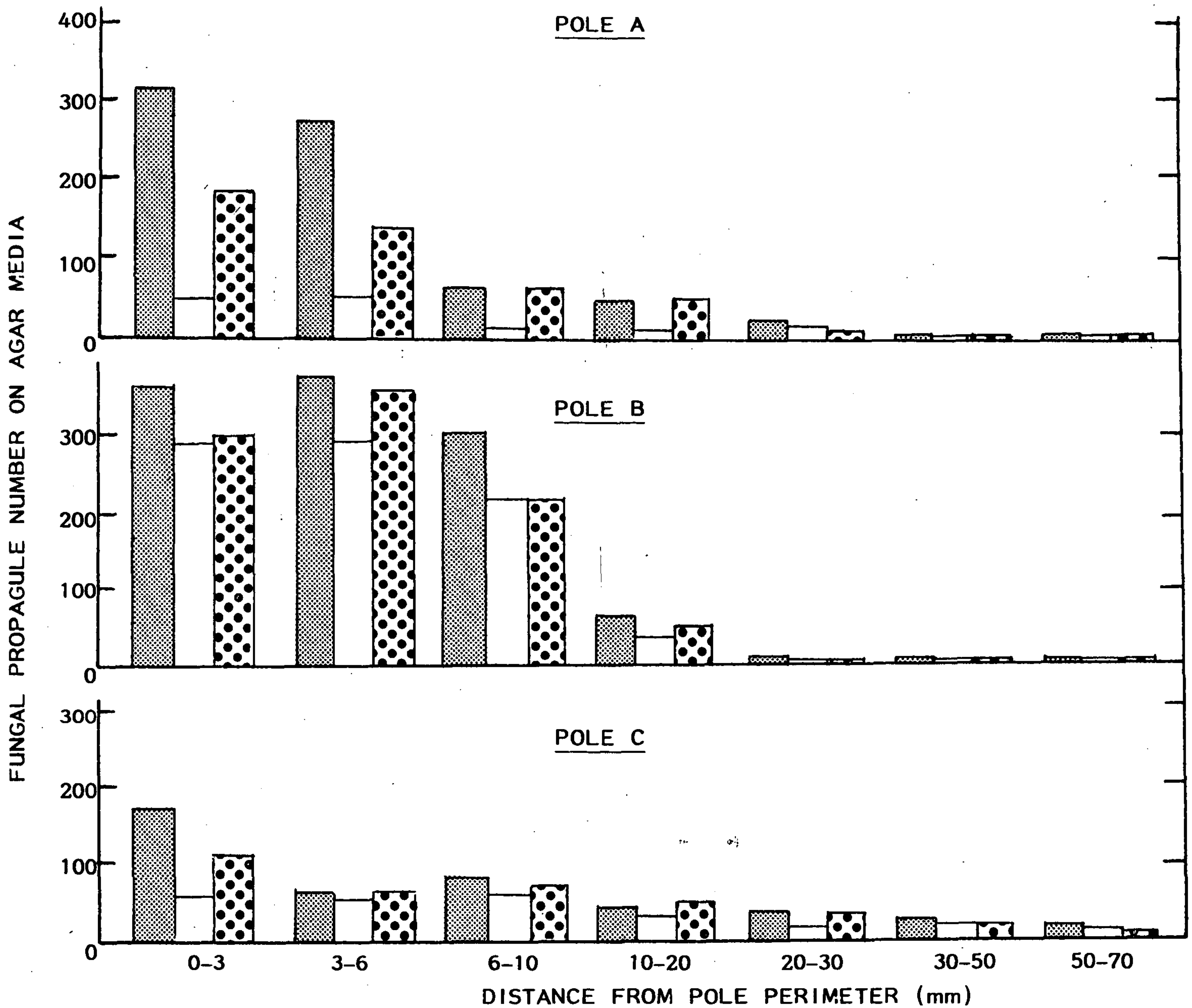
Fungal propagule counts per unit weight wood sawdust: Comparison of agar media.

Fungal propagule numbers per 0.01g sawdust (< 2mm mesh) were sampled along a radial transect of wood poles at the ground-line and plated onto three agar media. Counts shown were means obtained from three failed *Eucalyptus obliqua* poles.

▒ Glucose-asparagine agar, 4d incubation at 22°C.

□ Copper sulphate agar, 7d incubation at 22°C.

⁘ Cellulose agar, 12d incubation at 22°C.



POLE A
POLE B
POLE C
FUNGAL PROPAGULE NUMBER ON AGAR MEDIA
400
300
200
100
0
0-3
3-6
6-10
10-20
20-30
30-50
50-70
DISTANCE FROM POLE PERIMETER (mm)

Figure 13

Comparison of techniques for the assessment of the degree of wood degradation along a radial transect of soft-rotted Eucalyptus sp. hardwood poles.

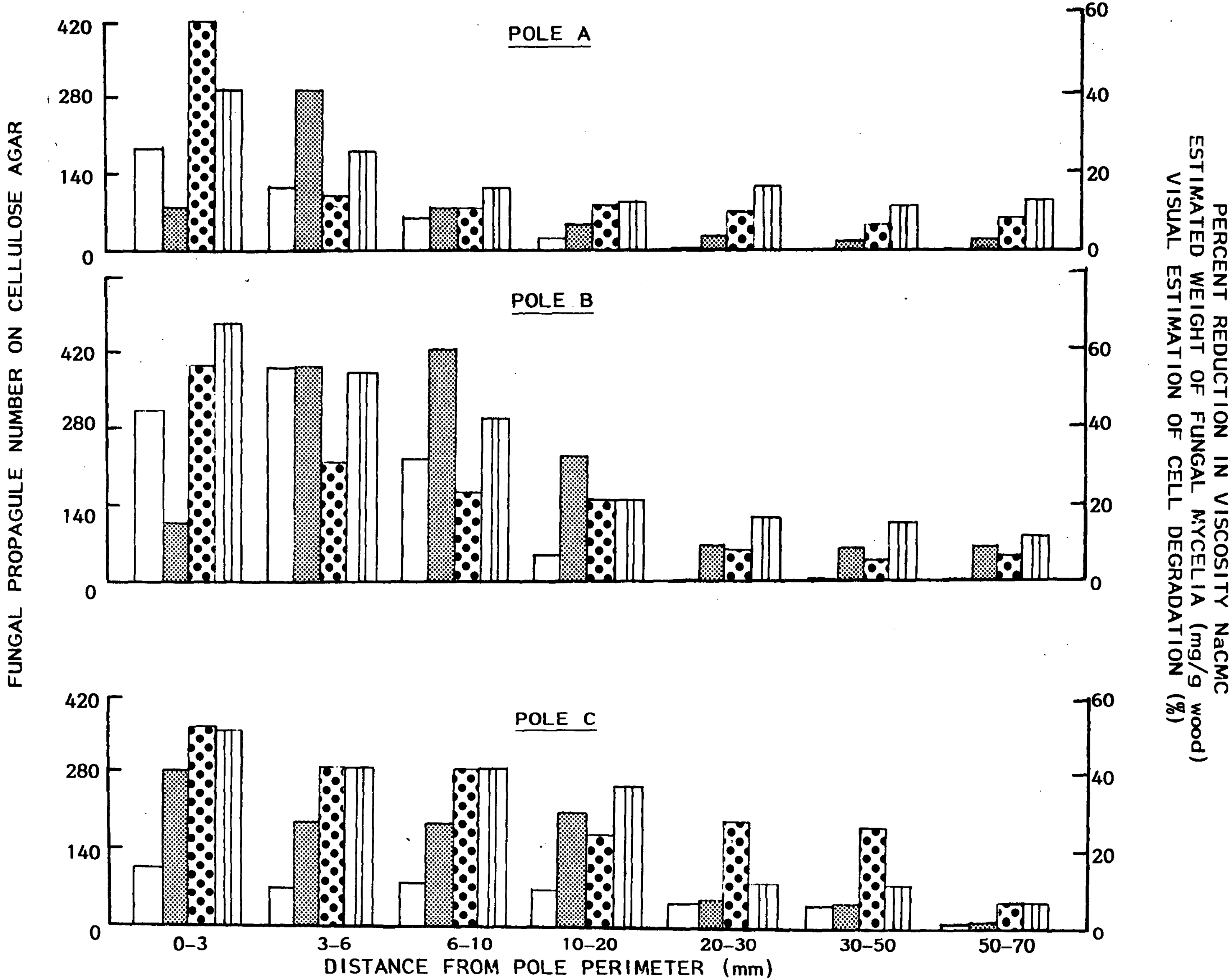
Fungal propagule numbers were means obtained from three replicate cellulose agar plates, 0.01g sawdust per plate. The incubation period was 12d at 22°C.

Cx-cellulase assay values were means of three replicate determinations. Sawdust samples (0.3g) were incubated in 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 45°C.

Mycelial biomass estimates were based on chitin assay values, assuming a mean 10% (w/w) chitin content of the fungal flora. Sawdust samples were hydrolysed in 5ml of 5N HCl for 20h at 80° C. Estimates were means of duplicate determinations.

Microscopic estimates of cell wall degradation.

All values were means of duplicate determinations.


POLE A
POLE B
POLE C
420
280
140
0
60
40
20
FUNGAL PROPAGULE NUMBER ON CELLULOSE AGAR
PERCENT REDUCTION IN VISCOSITY NaCMC
ESTIMATED WEIGHT OF FUNGAL MYCELIA (mg/g wood)
VISUAL ESTIMATION OF CELL DEGRADATION (%)
0-3
3-6
6-10
10-20
20-30
30-50
50-70
DISTANCE FROM POLE PERIMETER (mm)

3.6.1 Comparison of assay techniques using a pole stub trial at Grafton, N.S.W. (Koppers Aust. Pty. Ltd.)

The pole stub trial was emplaced by Koppers Aust. Pty. Ltd., to test the efficacy of pre-applied barriers at the groundline. *Eucalyptus maculata* stubs, positioned for two years, two months at the time of testing, were used to evaluate the Cx-cellulase assay, the fungal propagule count technique and the 6J Pilodyne(R) impaction device.

Both the Cx-cellulase assay and impaction determinations using the Pilodyne(R) showed highly significant differences ($p < 0.001$) in degradation between untreated and CCA-treated stubs (Table 10). However, only the enzyme assay produced further significant differences ($p < 0.001$) between CCA-treated stubs and stubs with additional treatments.

3.6.2 Comparison of assay techniques using a pole stub trial at Warrane, Tasmania

A CCA-treated *Eucalyptus* sp. pole stub trial inserted at Warrane, Tasmania, to test remedial treatments of soft-rotted poles, was used to compare the 6J and 10J Pilodynes(R), and to appraise the Cx-cellulase assay and the fungal propagule count technique.

Performances of the 6J (2.0mm pin diameter) and the 10J (2.5mm pin diameter) Pilodynes were evaluated by measurement of the impact resistances of 17 CCA-treated stubs. The regression of pin penetration into wood of the 6J Pilodyne on the pin penetration of the 10J device is detailed in Figure 14. A moderately close relationship between the pin penetrations of the two instruments was shown ($r^2 = 0.56$).

Table 10

Comparative assessment of Cx-cellulase activity with Pilodyne(R) determinations of wood impact strength, Grafton, N.S.W.

Treatment	Enzyme assay % reduction in viscosity NaCMC		Pilodyne (R) penetration (mm)	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.
Untreated (2 poles)	26.4	1.4	21.5	2.2
CCA-treated (5 poles)	6.2	1.6	10.2	0.6
CCA + Supplementary treatment (20 poles)	1.4	0.3	10.7	0.3

Sawdusted wood samples from duplicate ground-line cores (0-20mm) were incubated with 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) at 45°C. Determinations were means of duplicate samples.

Pilodyne penetration determinations were obtained using a 6J instrument with a 2mm pin diameter. Values were means of four assays per pole, taken at equidistant intervals around stubs at the ground-line.

Data for Table 10 are shown in Appendix 5.

Figure 14

Regression of the pin penetration into wood of a 6J, 2.0mm pin diameter Pilodyne (R) on the pin penetration of a 10J 2.5mm pin diameter Pilodyne.

Seventeen CCA-treated *Eucalyptus* sp. hardwood pole stubs of aged but sound condition, inserted at Warrane, Tasmania, for one year at the time of testing, were used for the comparison.

Wood moisture content was 15-20%.

Each value was the mean of four determinations.

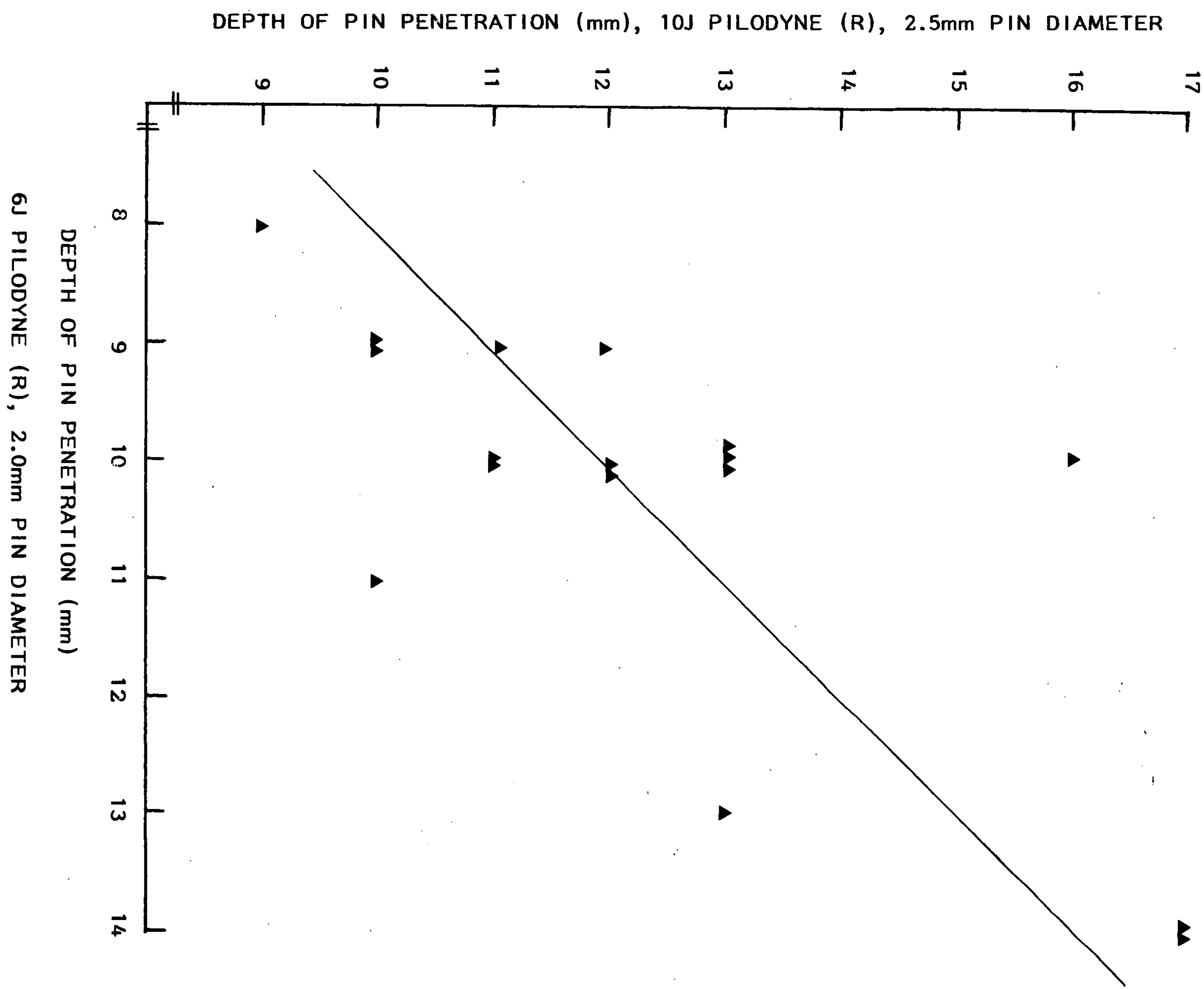
The regression equation was: $y = 1.53 + 1.04x$

$(r^2 = 0.56)$

Data for Figure 14 are shown in Appendix 6.

The mean penetration of the 6J Pilodyne $\bar{x} = 10.4$mm

The mean penetration of the 10J Pilodyne $\bar{y} = 12.4$mm


DEPTH OF PIN PENETRATION (mm), 10J PILODYNE (R), 2.5mm PIN DIAMETER
9
10
11
12
13
14
15
16
17
8
9
10
11
12
13
14
DEPTH OF PIN PENETRATION (mm)
6J PILODYNE (R), 2.0mm PIN DIAMETER

The efficacy of remedial preservatives was assessed by employment of the Cx-cellulase assay and the fungal propagule count technique. The control (untreated) stubs, emplaced 6 months after the remainder of the trial, showed an increase in activity between Samples (i) and (ii) when measured by both assay methods, and a decrease between Samples (ii) and (iii) [Figures 15(i)-15(iv)]. The wood moisture contents fell from 25-41% [Sample (ii)] to 8-15% [Sample (iii)].

Core samples from stubs treated with the Wolman bandage, the pentachlorophenol (PCP) bandage and the CSIRO-developed Blue 7 and Busan 30 Mark IV bandages were either sterile or showed slight microbial presence to 20mm depth, after two years emplacement with preservatives added [Figures 15(i)-(iv)]. Fungal propagule counts were higher in core samples from stubs treated with the Tanalith C bandage and bituminous paint than in samples from untreated controls.

The Cx-cellulase activity of samples from copper naphthenate and PCP-bandaged stubs was either approximately the same or lower than the activity of the control stubs in Sample (iii). The enzyme assays from samples of stubs treated with the Busan 30 and Blue 7 bandages showed markedly high levels of activity compared with the controls. Similarly, enzyme assays from stub samples tested with bituminous paint, creosote backfill and copper naphthenate were also high in the final sampling.

With the exception of activity levels in stubs treated with bituminous paint and PCP, the correlation of Cx-cellulase assay and the fungal propagule count was poor in this trial.

Figure 15

Comparative assessment of remedial treatments of soft-rot infected CCA-treated pole stubs (Warrane, Tasmania).

Fungal propagule counts were means of determinations obtained from cellulose agar plates, 0.01g sawdust per plate following incubation for 12d at 22°C.

Cx-cellulase assay values were means of 0.3g sawdust samples incubated in 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 50°C.

The results shown were means of either 10 samples (two cores per pole stub) in Samples (i) and (ii) or 25 samples (five cores per stub) in Sample (iii). Sample (i) was undertaken in January, 1979, 12 months after stub emplacement but just prior* to preservative application. Sample (ii) followed in August 1979, whilst Sample (iii) was undertaken 18 months later in February, 1981.

Pole stubs were either _E. globulus_ or _E. obliqua_ species woods. All assays were from core samples of 0-20mm wood depth.

*The Wolman bandage was applied after Sample (ii), August, 1979.

– Standard Error

– Preservative application

Data for Figures 15(i)-(iv) are shown in Appendix 7.

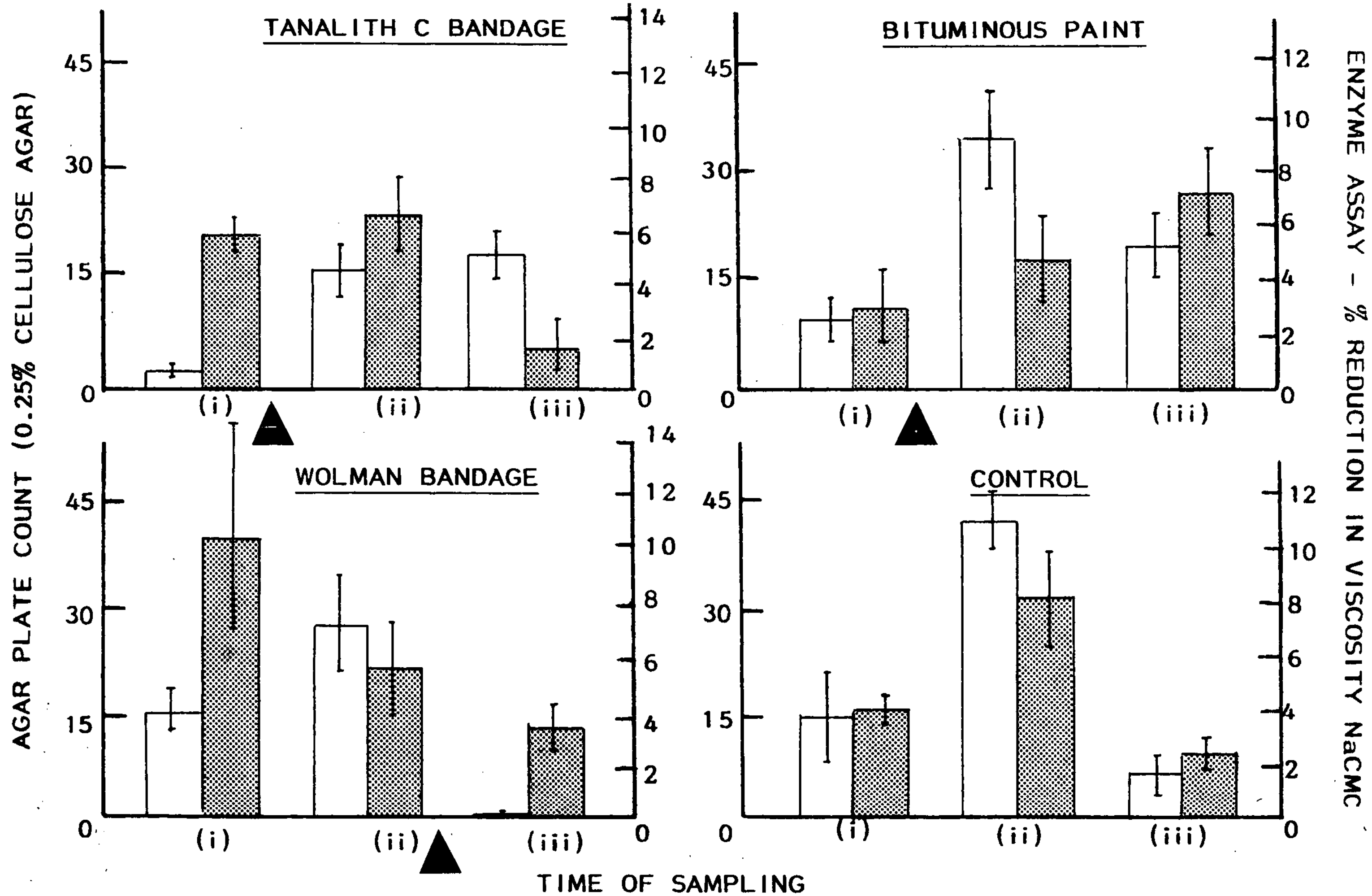

TANALITH C BANDAGE
BITUMINOUS PAINT
WOLMAN BANDAGE
CONTROL
AGAR PLATE COUNT (0.25% CELLULOSE AGAR)
ENZYME ASSAY - % REDUCTION IN VISCOSITY NaCMC
TIME OF SAMPLING
(i)
(ii)
(iii)
0
15
30
45
2
4
6
8
10
12
14

Figure 15(ii)

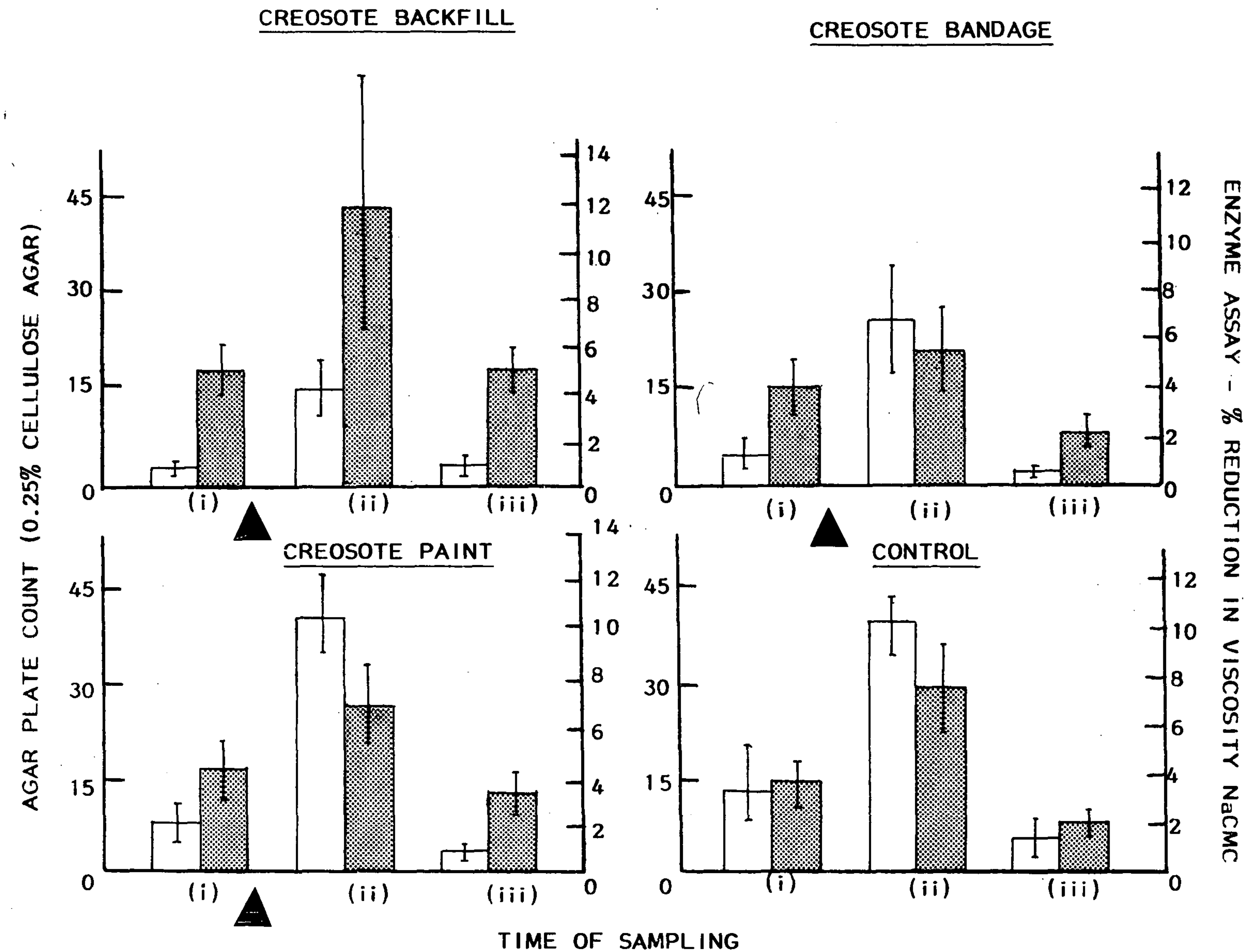
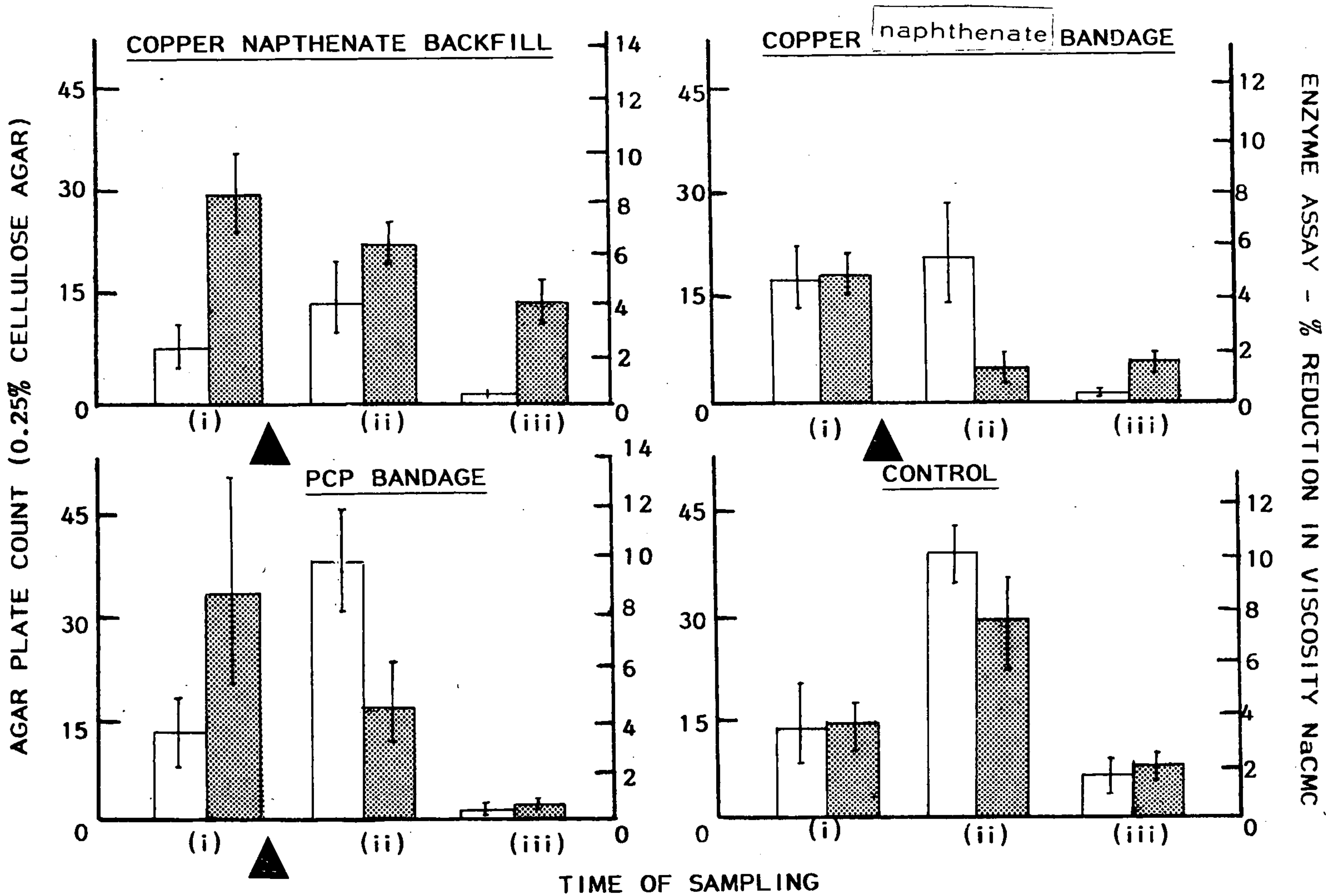
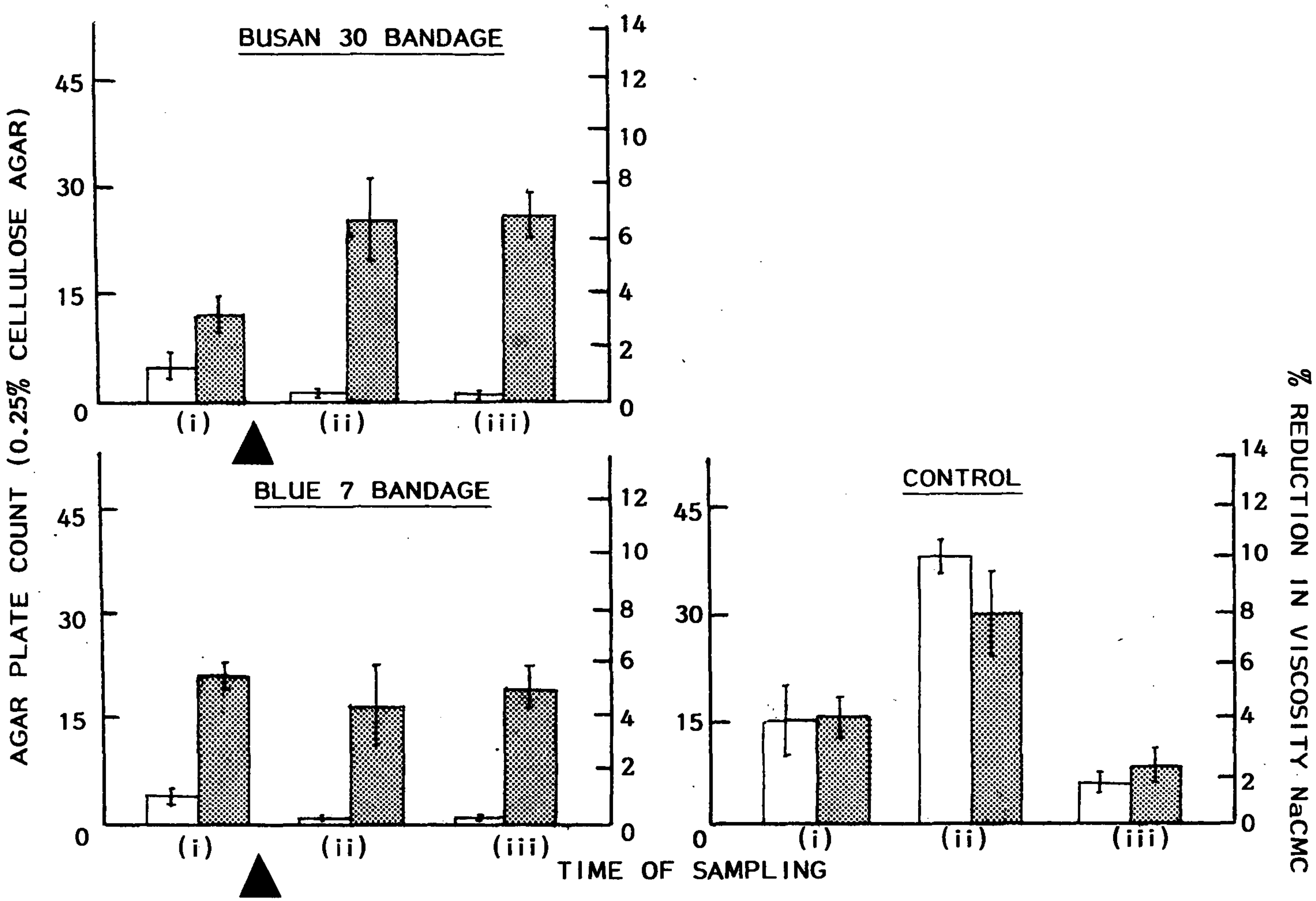

CREOSOTE BACKFILL
CREOSOTE BANDAGE
CREOSOTE PAINT
CONTROL
AGAR PLATE COUNT (0.25% CELLULOSE AGAR)
ENZYME ASSAY - % REDUCTION IN VISCOSITY NaCMC
0
15
30
45
0
2
4
6
8
10
12
14
(i)
(ii)
(iii)
TIME OF SAMPLING

Figure 15(iii)


COPPER NAPTHENATE BACKFILL
COPPER naphthenate BANDAGE
PCP BANDAGE
CONTROL
AGAR PLATE COUNT (0.25% CELLULOSE AGAR)
ENZYME ASSAY - % REDUCTION IN VISCOSITY NaCMC
(i)
(ii)
(iii)
TIME OF SAMPLING


BUSAN 30 BANDAGE
BLUE 7 BANDAGE
CONTROL
AGAR PLATE COUNT (0.25% CELLULOSE AGAR)
ENZYME ASSAY - % REDUCTION IN VISCOSITY NaCMC
TIME OF SAMPLING
(i)
(ii)
(iii)
0
15
30
45
2
4
6
8
10
12
14

The Warrane (Tasmania) pole stub site: A trial of remedial wood preservatives for treatment of soft-rot degradation

Plate 22 (top)

The trial site, looking west.

Plate 23 (middle)

An untreated (no preservative added) control pole stub. All stubs, both remedially treated and untreated, were CCA-treated *Eucalyptus* sp. woods.

Plate 24 (lower)

A Tanalith 'C' hessian bandage. Note the deterioration of the polyethylene outer covering.

The Warrane pole stub site (continued)

Plate 25 (top)

A pentachlorophenol (PCP) hessian bandage.

Plate 26 (middle)

A stub treated by backfilling with creosote.

Plate 27 (lower)

A C.S.I.R.O.-developed heat-shrink XLPE (Mark IV) bandage containing Blue 7 preservative. The Busan 30 bandages were of identical design.


55


84

The Warrane pole stub trial (continued)

Plate 28

A Wolman CFB bandage.

3.6.3 Comparison of assay techniques using a pole stub trial at Coff's Harbour, N.S.W.

The Cx-cellulase assay and the fungal propagule count technique were further appraised using a pole stub trial situated at Wedding Bells State Forest by the New South Wales Forestry Commission. The principal aim of the trial was to evaluate ground-line maintenance treatments for poles in the field.

Copper-chrome-arsenic-treated *Eucalyptus maculata* stubs with the CSIRO-developed Blue 7 and BFB Mark II bandages applied, had relatively high fungal propagule counts and Cx-cellulase activities compared with the other treatments emplaced after four years (Figure 16).

Both the Cx-cellulase assay and the fungal propagule count technique indicated comparatively low levels of activity in all creosote-treated stubs, in CSIRO Mark IV XLPE bandages (one year emplacement only), and in all *Pinus radiata* stubs.

3.6.4 Comparison of assay techniques using a sapwood stake trial at Grove, Tasmania

To evaluate the effectiveness of assay techniques for the estimation of wood degradation in short-term periods, a trial of various preservative treatments for *Eucalyptus obliqua* sapwood stakes was emplaced at Grove, Southern Tasmania, for 36 weeks. Where applicable, preservative retentions are listed in Appendix 9.

Upon retrieval, the untreated stubs, and stakes treated with boric acid/sodium tetraborate, had relatively high levels of fungal activity when assessed by visual inspection (Table 11), the Cx-cellulase assay, the chitin assay and the fungal propagule count on cellulose agar (Figure 17). Conversely,

Figure 16

Comparative assessment of ground-line maintenance treatments of pole stubs (Coff's Harbour, N.S.W.)

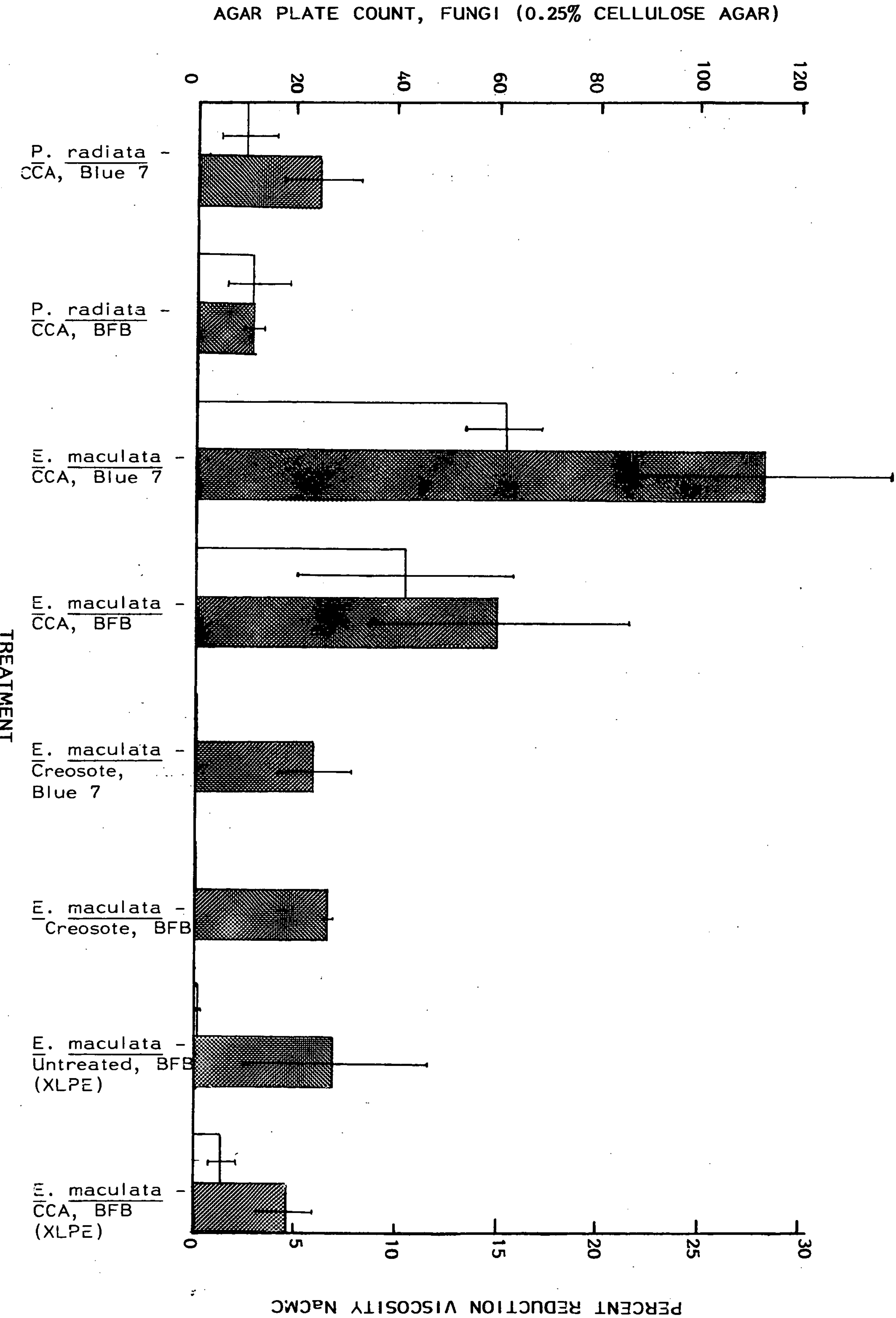
☐ Fungal propagule numbers were means obtained from three replicate determinations (three stubs, one determination per stub) on cellulose agar, 0.01g sawdust per plate. The incubation period was 12d at 22°C.

▩ Cx-cellulase assay values were means of three replicate determinations (three stubs, one determination per stub). Sawdust samples (0.3g) were incubated in 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 50°C.

Apart from the *E. maculata* - XLPE treatments which were emplaced for one year only, the pole stubs plus barriers had been in position for three years at the time of testing.

I - Standard error.

Data for Figure 16 are detailed in Appendix 8.


AGAR PLATE COUNT, FUNGI (0.25% CELLULOSE AGAR)
0
20
40
60
80
100
120
P. radiata - CCA, Blue 7
P. radiata - CCA, BFB
E. maculata - CCA, Blue 7
E. maculata - CCA, BFB
E. maculata - Creosote, Blue 7
E. maculata - Creosote, BFB
E. maculata - Untreated, BFB (XLPE)
E. maculata - CCA, BFB (XLPE)
TREATMENT
0
5
10
15
20
25
30
PERCENT REDUCTION VISCOSITY NaCMC

<u>The Coff's Harbour (N.S.W. Forestry Commission) pole stub trial of ground-line maintenance treatments for soft-rot degradation</u>

<u>Plate 29</u>

The trial site at Wedding Bells State Forest, looking north. Stubs emplaced were either <u>Eucalyptus</u> <u>maculata</u> or <u>Pinus</u> <u>radiata</u> woods.


PROTECTION OF POLES
AGAINST BIOLOGICAL DEGRADE

Table 11

Visual assessment of *Eucalyptus obliqua* sapwood stakes following 36 weeks field trial, Grove, Tasmania.

Preservative Treatment	Assessment rating of replicate stakes a	b	c	d	e
Creosote	–	–	–	–	8/8
Tanalith C Paste	–	–	–	1/8	7/8
$CuSO_4/K_2CrO_4$	–	–	–	6/8	2/8
$H_2BO_3/Na_2B_4O_7.10H_2O$	–	–	–	7/8	1/8
NaPCP	–	–	–	2/8	6/8
Bituminous Paint	–	–	2/8	5/8	1/8
Untreated Control		1/6	2/6	3/6	

Key: a – completely rotted

b – large pockets of rot, very soft

c – soft to touch in areas with scalpel

d – trace of softening in isolated areas

e – sound, very hard in all areas

Each preservative treatment was applied to 8 stakes; all of which were examined after emplacement.

Six untreated stakes only were assessed for control purposes.

Figure 17

Comparative assessment of selected wood preservatives for Eucalyptus obliqua sapwood stakes (Grove, Tasmania)

The stakes were inserted for 36 weeks before examination.

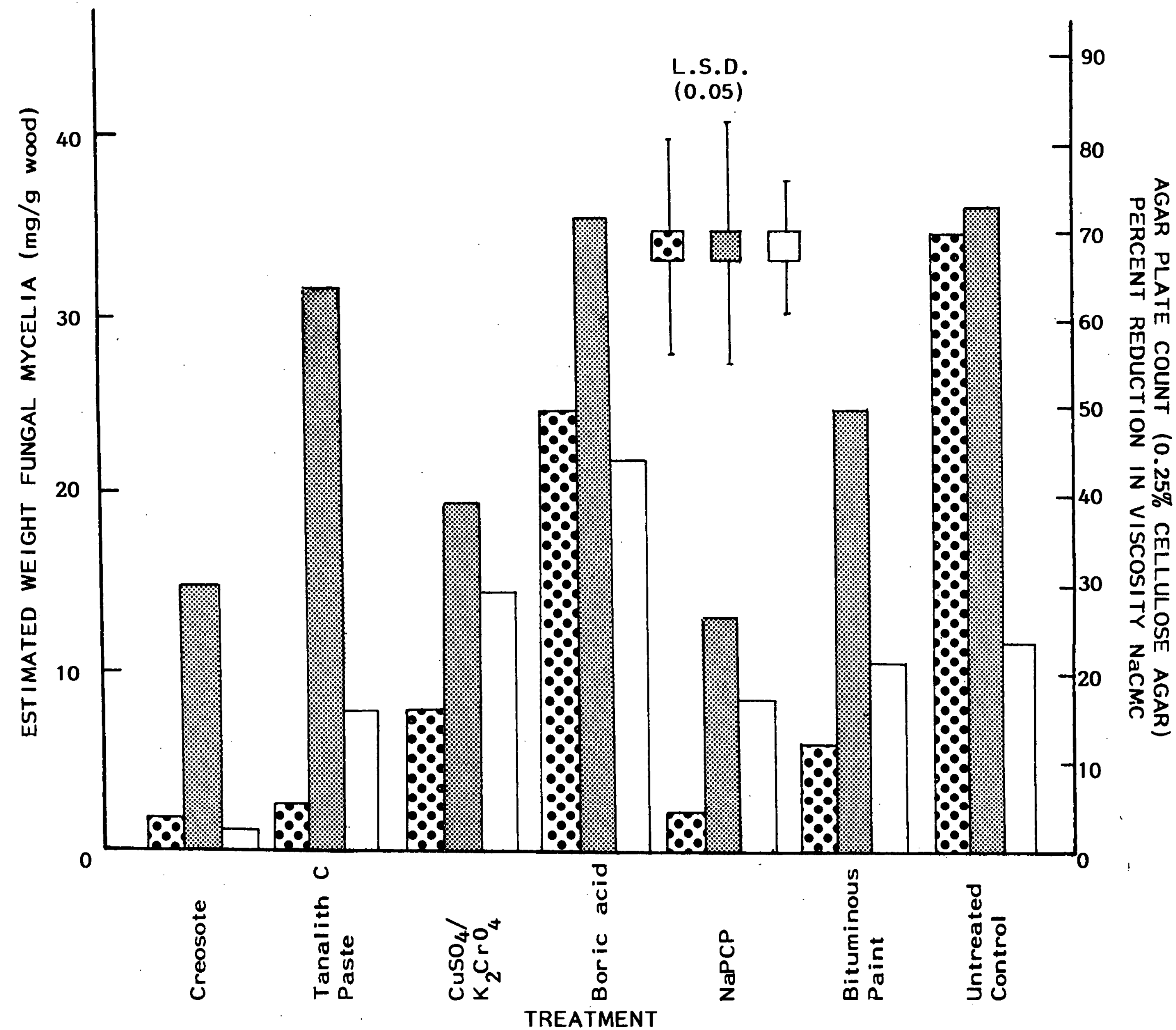
Mycelial biomass estimates were based on chitin assay values assuming a mean 10% (w/w) chitin content of the fungal flora. Sawdust samples (0.2g) were hydrolysed in 5ml of 5N HCl for 20h at 80°C.

Cx-cellulase assay values were means obtained from 0.3g sawdust samples incubated in 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 50°C.

Fungal propagule counts were means of determinations obtained from cellulose agar plates, 0.01g sawdust per plate. The incubation period before counting was 12d at 22°C.

The results shown were the means obtained from 8 replicate determinations (or 6 for control stakes).

Data for Figure 17 are shown in Appendix 9.



L.S.D.
(0.05)
ESTIMATED WEIGHT FUNGAL MYCELIA (mg/g wood)
0
10
20
30
40
AGAR PLATE COUNT (0.25% CELLULOSE AGAR)
PERCENT REDUCTION IN VISCOSITY NaCMC
0
10
20
30
40
50
60
70
80
90
Creosote
Tanalith C Paste
CuSO4/ K2CrO4
Boric acid
NaPCP
Bituminous Paint
Untreated Control
TREATMENT

the creosote-treated and NaPCP-treated stakes had low levels of microbial activity. These treatments were accordingly the most effective preservatives under test in this trial.

Conflicting evidence was obtained for stakes treated with Tanalith C (CCA) paste, with samples having high levels of enzyme activity but relatively low fungal propagule, biomass and visual degradation estimates. In addition, the fungal propagule numbers were lower in samples from untreated stakes compared with those from boric-acid treated woods. Trichoderma viride tended to overgrow other fungi on the agar plates with untreated sawdust added.

Comparisons of the Cx-cellulase assay, the chitin assay and the fungal propagule count technique for the assessment of wood degradation in the stakes are detailed in Figures 18 (i)-(ii). All three techniques demonstrated the relative effectiveness of the creosote and NaPCP treatments when compared with the untreated controls [Figure 18(i)]. Variable degrees of effectiveness were obtained for the remaining preservative treatments using the three methods.

An examination of the assay techniques using the best-performed treatment, creosote, as the standard, is made in Figure 18 (ii). All three assays showed highly significant differences between the creosote and the boric acid treatments, and between the creosote and control treatments. In comparison, the levels of microbial activity in the other preservative-treated woods were variable when assessed by the individual methods.

3.7 Studies on the stability of Cx-cellulases following microbial death

Due to the pronounced Cx-cellulase activity of sterile

Figure 18

Comparison of assay techniques using the L.S.D. Test (i)

Differences in levels of microbial activity between treated and untreated control stakes emplaced at Grove, Tasmania, as determined by the Cx-cellulase assay, the chitin assay and the fungal propagule count. The comparison was made using the Least Significant Difference (L.S.D.) statistical test.

[For differences between means of the assay techniques to be statistically significant at an chosen level of probability (e.g. $p < 0.001$), the observed difference between treatment and untreated control means exceeded the calculated LSD at that level.]

Comparison of treatment means with untreated control means, Grove stake trial - L.S.D. basis

	Creosote	Tanalith 'C' Paste	$CuSO_4$/ K_2CrO_4	H_3BO_3/ $Na_2B_4O_7.10H_2O$	NaPCP	Bituminous Paint
Enzyme assay	$p < 0.001$	ns	$p < 0.01$	ns	$p < 0.001$	$p < 0.05$
Mycelial biomass estimate - chitin assay	$p < 0.001$	$p < 0.001$	$p < 0.001$	ns	$p < 0.001$	$p < 0.001$
Fungal propagule count (on 0.25% cellulose agar	$p < 0.001$	ns	ns	$p < 0.01$*	ns	ns

*Treatment mean higher than control mean.

ns = no significant difference ($p > 0.05$).

Figure 18

Comparison of assay techniques using the L.S.D. Test (ii)

Differences in levels of microbial activity between the creosote treatment and other stake treatments, Grove, Tasmania, as determined by the Cx-cellulase assay, the chitin assay and the fungal propagule count. The comparison was made using the Least Significant Difference (L.S.D.) statistical test.

[For differences between means of the assay techniques to be statistically significant at a chosen level of probability (e.g. $p < 0.001$), the <u>observed</u> difference between creosote treated and other treatment means exceeded the calculated L.S.D. at that level.]

Comparison of creosote treatment means with other treatment means, Grove stake trial - L.S.D. basis

	Tanalith 'C' Paste	$CuSO_4$/ K_2CrO_4	H_3BO_3/ $Na_2B_4O_7.10H_2O$	NaPCP	Bituminous Paint	Untreated Control
Enzyme assay	$p < 0.01$	ns	$p < 0.001$	ns	ns	$p < 0.001$
Mycelial biomass estimate - chitin assay	ns	ns	$p < 0.001$	ns	ns	$p < 0.001$
Fungal propagule count (on 0.25% cellulose agar)	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$

ns = no significant difference ($p > 0.05$).

The Grove (Tasmania) Eucalyptus obliqua sapwood stake trial of wood preservatives

Plate 30 (top)

The trial site looking north-west. Each stake had dimensions of 300 x 40 x 15mm.

Plate 31 (lower)

Eucalyptus obliqua sapwood stakes not inserted at the Grove site. The preservative treatments were (from left to right): Tanalith 'C' paste, creosote and untreated control.

The Grove E. obliqua sapwood stake trial (continued)

Plate 32 (top)

E. obliqua stakes not inserted at the site. The preservative treatments applied were (from left to right): $H_3BO_3/Na_2B_4O_7.10H_2O$, $CuSO_4/K_2CrO_4$, sodium pentachlorophenate, bituminous paint.

Plate 33 (middle)

E. obliqua stakes after 36 weeks emplacement at the Grove site. The preservatives were (from left to right): $H_3BO_3/Na_2B_4O_7.10H_2O$, Tanalith 'C' paste, untreated control.

Plate 34 (lower)

E. obliqua stakes after 36 weeks emplacement at the site. The preservatives were (from left to right): Sodium pentachlorophenate, creosote, $CuSO_4/K_2CrO_4$, bituminous paint.

or near sterile wood samples from the Wolman bandage, Blue 7 and Busan 30 bandages on <u>Eucalyptus</u> <u>obliqua</u> pole stubs inserted at Warrane, Tasmania (Figures 15(i)-(iv)], an investigation was made into the leachability and longevity of the enzymes in wood after microbial death.

To study these aspects, an organic solvent was needed to sterilise the wood without having a detrimental effect on Cx-cellulases. After testing of 10 solvents (Table 12), both n-butanol and chloroform had the desired properties. Chloroform was selected as the solvent for use in this study.

Cx-cellulases were leached from sterile, soft-rotted wood blocks positioned in water-saturated vermiculite (Figure 19). Enzyme activity in the "leachate" was still measurable after 75d incubation in sterile conditions at 22 C.

After 75d incubation, the wood blocks themselves were tested for Cx-cellulase activity. Blocks wetted, incubated in sterile conditions for 75d and redried to the same moisture content as the dry blocks (14-17%) had a mean Cx-cellulase activity of 16.7% reduction in viscosity of NaCMC (S.E. = 1.2%), whereas blocks kept dry had a mean activity of 53.1% (S.E. = 7.4%). The activity of the 'wetted' blocks was 31% of control (dry) blocks.

Table 12

Sieved sawdust was placed in bottles and saturated with a range of solvent vapours for 36h at 22°C.

Both fungal and bacterial agar plate counts were means obtained from duplicate plates, 0.01g sawdust per plate.

Fungal propagule determinations were made using glucose-asparagine agar, incubated for 4d before counting.

Bacterial colony counts were determined using tryptic yeast extract agar (TYE) plates, incubated for 4d before counting.

Cx-cellulase assay values were means of duplicate determinations. Sawdust samples (0.3g) were incubated in 10ml of 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 45°C.

Table 12

Studies on the stability of Cx-cellulases in wood following microbial death.

Efficacy of selected organic solvents as sterilising agents.

Solvent	Viable fungal propagule count	Viable bacterial count	Percent reduction in viscosity NaCMC	Final rating
Acetone	0	12	76.0	**
Ammonia	0	2	27.3	*
n-butanol	1	12	89.5	***
Chloroform	1	9	88.8	***
Cyclohexane	18	>> 200	87.6	*
Cyclohexanone	39	78	87.8	*
Absolute ethanol	0	9	68.5	**
Formaldehyde	NT	NT	18.8	*
Glutaraldehyde	>> 50	19	86.9	*
Propylene oxide	0	3	1.5	*
Control (untreated sawdust)	>> 50	>> 200	89.0	-

NT = not tested

* = poor

** = fair

*** = good sterilising agent, little effect on Cx-cellulase activity.

Figure 19

Studies on the stability of Cx-cellulases in wood following microbial death

Leaching of Cx-cellulases from soft-rotted wood blocks into water.

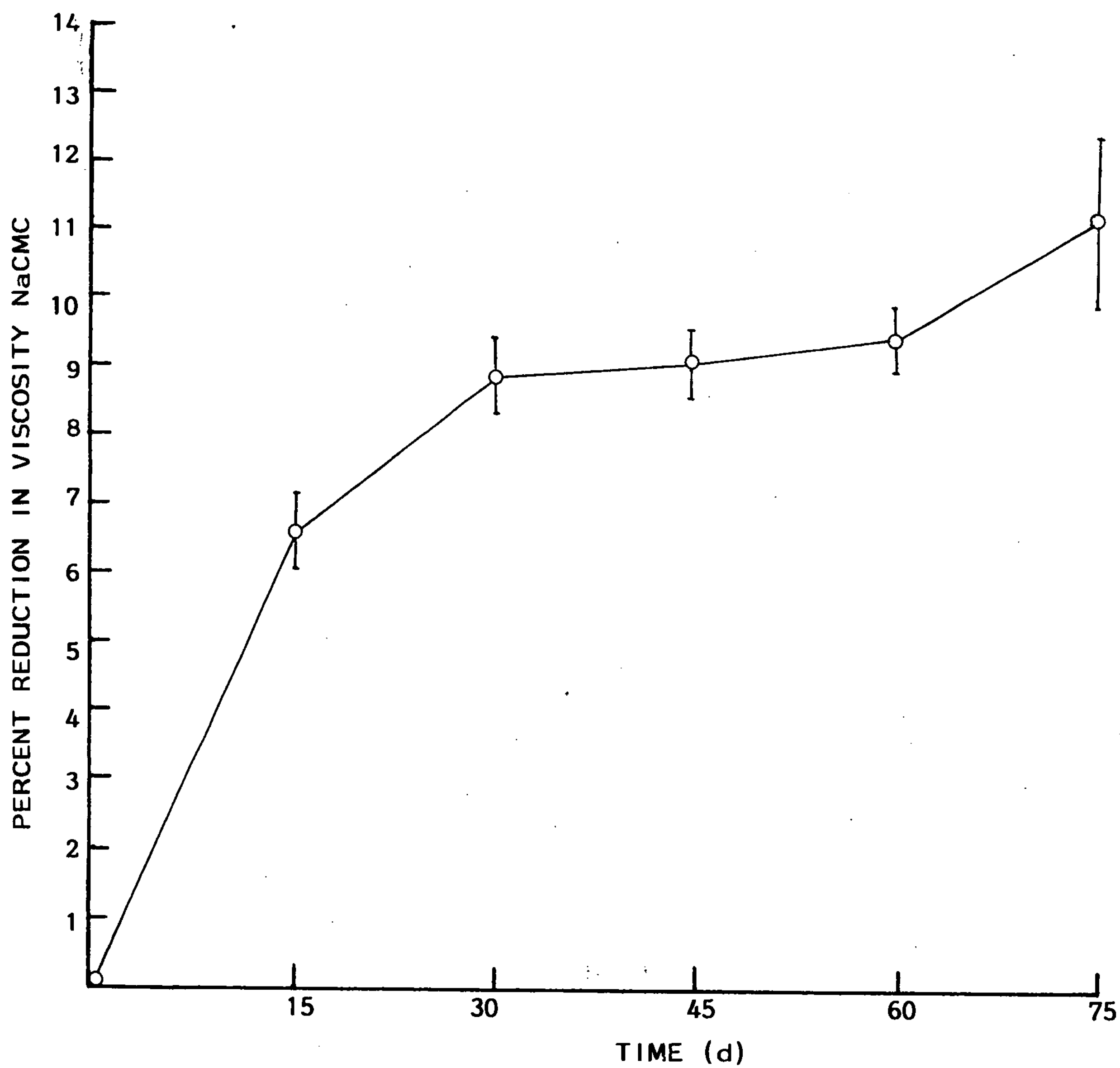
Three sterilised, soft-rotted *Eucalyptus* sp. blocks of 100 x 15 x 20mm approximate dimensions were placed in glass jars containing 30g vermiculite plus 270ml water. The jars were incubated for 75d at 22^{o}C.

At 15d intervals, 1ml aliquots of 'leachate' surrounding the woods were added to 10ml of 0.5% NaCMC in 0.1M acetate buffer (pH 5.5) and incubated for 1h at 50^{o}C.

Each value was the mean of eight determinations (4 flasks, 2 determinations per flask).

I - Standard error

Data for Figure 19 is shown in Appendix 10.


PERCENT REDUCTION IN VISCOSITY NaCMC
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
30
45
60
75
TIME (d)

DISCUSSION

4. DISCUSSION

4.1 Microorganisms Isolated from Soft-Rotted CCA-Treated Woods in Tasmania

The predominant fungus isolated from Tasmanian CCA-treated soft-rotted Eucalyptus sp. woods was the Hyphomycete Phialophora mutabilis. Line and Cruickshank (1979), Leightley (1980b) and Leightley, Francis and Johnstone (1980) also found P. mutabilis to be a dominant organism degrading woods in ground contact in various Australian localities. Species belonging to the genus Phialophora were established by Henningsson and Nilsson (1976) to be dominant organisms in Scandinavian CCA-treated poles; however, P. mutabilis was infreqently isolated in their investigation.

Other sap-staining or known soft-rotting organisms often isolated from woods in the present study and encountered by previous workers in related investigations, were Trichoderma viride (Merrill and French, 1966; Greaves, 1972a; Henningsson and Nilsson, 1976; Leightley, Francis and Johnstone, 1980); Oidiodendron griseum and Doratomyces microsporus (Henningsson and Nilsson, 1976); Paecilomyces varioti (Greaves, 1972a; Leightley, 1978); Fusarium decemcellulare (Greaves, 1972a).

Chaetomium globosum, a test fungus often used for soft-rot decay studies and laboratory testing of wood preservatives, was not isolated in this study. No reported isolations of this organism appear to have been made in Australia. Henningsson and Nilsson (1976) noted that it was rarely found in Scandinavian CCA-treated woods.

Previous workers have isolated bacteria from wood in soil belonging to the same genera as the cellulolytic and pectolytic organisms isolated from CCA-treated *Eucalyptus* sp. woods and identified in this study (Tables 5-6). Greaves (1973) isolated *Bacillus megaterium*, *Flavobacterium* (2 species) and three *Cellulomonas* spp. from CCA-treated *Eucalyptus* sp. and untreated woods in Australia or New Guinea. All isolates excepting one *Flavobacterium* sp. were cellulolytic in culture. Willeitner, Schmidt and Wollenberg (1977) isolated *Cellulomonas*, *Flavobacterium* and *Bacillus* spp. from woods or bagasse in soil. The *Cellulomonas* sp. could degrade pectin, hemicellulose, cellulose and wood shavings. Thayer and Murray (1977) found a cellulolytic strain of *Bacillus megaterium* which could degrade mesquite wood. Schmidt (1980) tested isolates of *Bacillus megaterium* (2 strains), *Cellulomonas* (2 species), *Nocardia* (5 species) and *Streptomyces* (5 species) for activity on European beech and Scot's pine sapwood blocks. None of the bacteria attacked the unlignified cell walls of these woods.

4.2 Aspects of Wood Substrate-degrading Capacities of Isolated Microorganisms

When glucose was used as the major carbon source for fungal enzyme production, either absence of cellulose in the medium, or catabolite repression of cellulase induction (Hulme and Stranks, 1970) may explain the failure of some isolates to elaborate Cx-cellulases (Table 1).

All fungi with demonstrable Cx-cellulase (NaCMC-ase) activity in cultures with glucose, micro-crystalline cellulose or cotton wool (crystalline cellulose) as carbon sources, also cleared Walseth or acid-swollen cellulose and released dye from RBBR-cellulose (Table 1). However, disparate levels of cellulolytic activity were noted when examined by the three techniques. For example, whilst Fusarium decemcellulare was strongly cellulolytic when assessed by all techniques, Chaetomium globosum had high levels of Cx-cellulase and dye release activity, but its capacity to degrade Walseth cellulose, in comparative terms, was low.

Although Nilsson (1973) considered the clearing of Walseth cellulose to be a useful method of assessing fungal cellulolytic activities due to the necessity of C1 factor (or cellobiohydrolase) production, it is possible that C1 and Cx enzymes acting individually can both degrade the substrate (Berghem and Pettersson, 1973; Enari and Markkanen, 1977; Wood and McCrae, 1977, 1978; Highley, 1980). This may be dependent on the degree of cellulose crystallinity (Tanaka, Taniguchi, Morita, Matsuno and Kamikubo, 1979). The above alternatives could explain the differences in levels of cellulolytic activity noted in this study. If production of the C1 factor, needed for complete degradation of crystalline cellulose, was necessary to exhibit a synergistic response in the clearing of acid-swollen cellulose, then organisms including Fusarium decemcellulare, Phialophora mutabilis (strain B) and Polystictus versicolor were able to synthesise greater quantities of the C1 factor than most of the other test fungi in culture.

Alternatively, the variabilities in clearing of Walseth cellulose agar deeps compared with other cellulase assays may be due to differences in Cx-cellulase molecular weights, with smaller molecules diffusing to greater depths than larger enzymes.

All fungi with marked capacities to release dye from RBBR-cellulose, also produced high Cx-cellulase levels in culture (Table 1). Therefore, the Cx-cellulases producing the viscosity reductions of NaCMC may also be able to release dye from RBBR-cellulose. However, no apparent correlation existed between the amounts of RBBR-dye released by fungi and the depths of clearing of Walseth cellulose.

Differences in the cellulase activities of fungi were undoubtedly responsible for the sharp or diffuse clearing of Walseth cellulose (Table 1). A sharp border between the fungal-degraded and undegraded acid-swollen cellulose could indicate a greater range of enzymes produced, cellobiohydrolases, Cx-cellulases or both, than a diffuse boundary. An alternative hypothesis is that a sharp boundary may indicate strong binding of enzymes to the substrate until release by dissolution.

It would seem that several assay methods should be utilized in combination when examining fungal cellulolytic activity in wood degradation studies.

All cellulolytic fungal isolates tested had measurable hemicellulase activity (Table 2) using extracted and purified <u>Eucalyptus obliqua</u> xylan and mannan, and larch (<u>Larix</u> sp.) xylan as substrates. Gascoigne and Gascoigne (1960), Lyr and Novak (1961), Nilsson (1974b) and Leightley and Eaton (1977) found similar correlations between cellulase and hemicellulase production by various Fungi Imperfecti and Ascomycetes.

Eucalyptus obliqua mannan and to a lesser extent larch xylan were commonly utilized by the test fungi, but clearing of *E. obliqua* xylan was generally indeterminate, with the fungal mycelia tending to grow in advance of any developing substrate clearing zone. Most fungal isolates examined were probable degraders of *E. obliqua* xylan, but doubt existed. To aid in assessment, Flannigan and Gilmour (1980) flooded xylan-containing agar plates with ethanol to precipitate the undegraded substrate when testing for enzyme action.

The production of numerous hemicellulases by fungi has been previously reported by Domsch and Gams (1969), Takahashi and Nishimoto (1973) and Highley (1976). A relationship between the preferred host substrate (hardwoods rather than softwoods for soft-rot fungi) and the utilization of the predominant hemicellulose (xylan in hardwoods rather than mannan from softwoods) has been proposed (Keilich, Bailey and Liese, 1970; Takahashi and Nishimoto, 1973). In this study, extracted *Eucalyptus obliqua* (hardwood) mannan was readily metabolized by the majority of test fungi in comparison with larch and possibly *E. obliqua* xylan. Both xylans and mannans appear to be degraded by Tasmanian fungal isolates from soft-rotted *Eucalyptus* sp. woods.

Fungal amylase and pectic enzyme activities were examined using polyacrylamide gel electrophoresis. With this technique, the range of fungal enzymes can be examined. Different enzyme (protein) mobilities or migration distances (Rf values) allowed comparison of enzymes both within and between species of microorganisms. The acrylamide acts as a molecular sieve; accordingly, enzyme mobility is dependent on molecular weight, size (shape) and electric charge (Gordon, 1969).

A wide range of fungal alpha-amylases were detected after iodine staining (Bird and Hopkins, 1954; Robyt and French, 1967) (Table 3, Plate 10). The mould or sap-staining fungi tested (*Aspergillus fumigatus*, *Penicillium frequentans*, *Aureobasidium pullulans* and *Trichoderma viride*) all produced at least two distinct amylases, whilst fungi known to be soft-rotting organisms (*Chaetomium globosum*, *Doratomyces microsporus*, *Oidiodendron griseum* and *Phialophora mutabilis*; Butcher, 1968b; Line and Cruickshank, 1979) produced only one starch-degrading enzyme.

Although wood generally contains less than 5% pectin by weight (Meier, 1964; Herrick and Hergert, 1977), a range of pectic enzymes were elaborated by fungi isolated from Tasmanian *Eucalyptus* sp. woods (Table 3, Plate 11). A series of pectic enzymes were produced by some individual fungi. *Aureobasidium pullulans*, a sap-staining organism, produced two polygalacturonases in culture, whilst the soft-rotting organism *Oidiodendron griseum* synthesised four pectic enzymes including one pectin esterase. No correlations between variations in pectic enzyme production and sap-staining or soft-rotting abilities could be drawn in this study.

Demonstrable laccase production by three imperfect fungi (*Cephalosporium acremonium*, *Fusarium decemcellulare* and *Graphium rigidum*) confirmed reports by workers including Drew and Kadam (1978), Crawford and Crawford (1980) and

Norris (1980) who have noted the abilities of some lower fungi to elaborate lignin-degrading enzymes.

The presence of cellulolytic activity in these tests indicated only the potential for fungal cellulase synthesis and action in wood. Tasmanian fungal isolates which were potent producers of Cx-cellulase in culture did not necessarily cause large weight losses of *Eucalyptus obliqua* wood blocks in a 16 week incubation period (Table 4). For example, *Trichoderma viride* was highly cellulolytic in culture; a fact noted by other workers including Berghem and Pettersson (1973), Sternberg (1976) and Linko (1977). However, in comparison with other test isolates examined in this study, minimal sapwood weight losses were produced by this organism. This supports the work of Line and Cruickshank (1979, *Trichoderma* sp. = *T. viride*) and Leightley (1980b) who found that cellulolytic isolates did not necessarily produce soft-rot cavities (Type 1) in *Eucalyptus* sp. woods. Henningsson and Nilsson (1976), however, did observe some erosion attack (Type 2) by a *T. viride* isolate.

Obviously assessment of cellulase activity using pure culture conditions does not predict the potential of fungi to attack wood in ground contact. If cellulases are synthesised in wood by fungi including *T. viride*, the ability to overcome the occlusion of cellulose by lignin may determine whether degradation by an individual organism proceeds (Jutte and Wardrop, 1970; Polcin and Bezúch, 1977). Variations in fungal physiology such as the intolerance of *T. viride* to low oxygen tension (Garrett, 1963), will also affect the organism's ability to degrade wood.

Conversely, Paecilomyces variori produced a degree of E. obliqua sapwood degradation (0.42-2.79% weight loss) but exhibited no detectable cellulase activity in pure culture. Even though the sapwood weight losses in this study were low (0.42-5.65% for imperfect fungi), it is possible that P. variori was a non-cellulolytic soft-rot fungus (Nilsson, 1974b). If so, the conditions used for cellulase synthesis in pure culture were inappropriate for this organism (Basu and Ghose, 1960; Nilsson, 1974b). Lignified substrates may stimulate cellulase activity by some fungi (Nilsson, 1974c; Hofsten, 1975; Vohra, Shirkot, Dhawan and Gupta, 1980); P. variori may need such a stimulus.

Phialophora mutabilis, Doratomyces microsporus and Graphium rigidum had minimal cellulolytic activity when assessed by clearing of Walseth cellulose, but caused comparatively high weight losses of E. obliqua sapwood blocks. If, as Nilsson (1973) has suggested, the fungal degradation of both Walseth cellulose and wood cellulose involves production of C1 factor or factors, then, with the exception of Polystictus versicolor, the correlation between levels of the two cellulose degradations has been poor in this study.

The predominant soft-rotting organism isolated from Tasmanian eucalypt woods in Tasmania, Phialophora mutabilis could elaborate in culture: cellulases (Cx and probably C1 enzymes) amylases (at least one enzyme), xylanases, mannanases and pectic enzymes. In contrast, the sap-staining fungus Aureobasidium pullulans, also commonly isolated from CCA-treated poles in Tasmania, produced only amylases and pectic enzymes.

Bacteria isolated from Tasmanian woods were shown to possess a range of wood-degrading enzymes. For example, Cellulomonas sp. S10N attacked both crystalline and carboxymethyl celluloses, larch xylan, starch and pectin, whilst Cellulomonas sp. S8N produced laccases in addition to cellulases, xylanases and pectic enzymes. Bacillus megaterium has the potential to elaborate NaCMC-ase, xylanase, amylase and pectic enzymes in wood.

Pectic enzyme production was established for approximately 35% of the isolates examined. Increases in wood permeability resulting from degradation of pit membranes has been attributed to the production of pectic enzymes by bacteria (Knuth, 1964; Johnson, 1979).

Whilst the examination was not detailed, microscopic inspection of bacterial attack on E. obliqua wood after 14d incubation revealed little structural damage to intact cell walls (Plates 12-17), although the cutting of medullary ray cell walls during microtomy has probably exposed previously inaccessible wall contents to degradation (Plate 14). Greaves (1973) found only minor wall destruction in his study of bacterial wood degradation. Holt and Gareth-Jones (1978) and Schmidt (1978, 1980) believe that a degree of delignification is required before bacteria will attack intact cell walls. Visible growth by Nocardia rugosa and Streptomyces sp. in parenchyma cells (Plates 12-13) suggested that, in the period of attack studied, degradation of starch or easily metabolised non-fibrillar material was occurring. Rossell, Abbott and Levy (1973) in their review, noted that the first structural change produced in wood by bacteria seems to be decomposition of materials stored in (ray) parenchyma cells.

Scanning electron micrographs of bacterial attack on _E. obliqua_ sapwood did not show any discernible wood degradation (Plates 16-17), although the Actinomycete _Streptomyces_ sp. had colonised the microtomed surface. The strands of slime visible (Plate 17) may be the 'glycocalyx' observed by Vance, Stanley and Brown (1979). This material could aid bacterial adhesion, enzyme concentration and conservation.

4.3 Aspects of the Ecology of Microorganisms in Eucalyptus sp. Woods

The examination of microbial colonisation of untreated _Eucalyptus obliqua_ sapwood stakes in ground contact (3.1.4) was not extensive, so few firm conclusions can be drawn. However, imperfect fungi (including known soft-rotting organisms), bacteria and Basidiomycetes were _all_ represented amongst the initial invaders since isolates from the three groups were obtained after 12 weeks ground emplacement.

More exhaustive studies by Butcher (1968b) and Banerjee and Levy (1971) favour a classical succession similar to the following: bacteria → primary moulds → soft-rot fungi → secondary moulds → Basidiomycetes. However, both Butcher (1968b) and Greaves (1972a) did observe unidentified Basidiomycetes amongst the early colonising organisms of woods in ground contact, whilst Sharp (1975) formed an alternative hypothesis as he found an absence of succession sequences from initial colonisation of wood veneers to almost complete substrate degradation. It is difficult from this study to determine which of these alternatives is correct.

Included among the imperfect fungi isolated were known soft-rot fungi Oidiodendron griseum (12 weeks insertion) and Phialophora mutabilis (24 weeks and a dominant organism at 36 weeks emplacement) were identified. The mould Trichoderma viride was presumably an early wood coloniser as it was predominant on sawdusted agar plates after incubation. Merrill and French (1966) and Butcher (1968b) noted similar isolation frequencies of this organism from woods in ground contact. In this investigation, T. viride produced a wide range of potential wood-degrading enzymes in culture; a major aspect of competitive saprophytic ability (Garrett, 1963). To further aid its growth in competition with other organisms, it can elaborate antibiotics (Dennis and Webster, 1971; Toole, 1971b; Kelly, Morton and Edmunds, 1981). Yet, rapid growth in culture conditions may not indicate a corresponding activity in wood, as organisms including T. viride could obscure the presence of other fungi (Butcher, 1968b; Dwyer and Levy, 1976).

Positive regressions observed between propagule estimates of fungi in CCA-treated wood and bacterial colony numbers, both on agar media (Figure 2), indicate that a degree of mutualism may exist between the two groups. That is, the presence of one group did not antagonise the other. Variations in fungal propagule numbers/unit sawdust tended to be reflected in similar variations in estimated bacterial populations. Blanchette and Shaw (1978) found similar associations between bacteria, yeasts and basidiomycetous fungi during wood decay.

A possible explanation would involve the opening-up of inner wood cells by anastomizing fungal hyphae, allowing bacteria to invade. These bacteria may produce co-factors for fungal growth (Henningsson, 1967; Liese and Karnop, 1968).

The regression line for both samples tested in Figure 2 cut the Y axis at a positive value. Bacteria may be present in wood prior to fungal colonisation in the ground (Shortle and Cowling, 1978a). More importantly, the fungal mass in wood would have been localised due to discrete fungal hyphae. Bacteria may be more dispersed, occurring on more particles after sawdusting. Hence, the same biomass of fungi and bacteria would generate greater bacterial colony numbers per unit of sawdusted wood on agar media than fungi. However, bacterial populations in degraded wood samples would still be underestimated to a significant degree. Clumping of cells in microcolonies would lead to single colonies being produced on agar plates from a single sawdust particle. Vigorous blending of sawdust in liquid media, for example, would probably give a more realistic appraisal of bacterial numbers in wood samples.

In view of the above comments, a bacterial biomass of moderate size existed in untreated *Eucalyptus obliqua* wood stakes. The decline in estimated numbers from 1.2×10^4 cells/g sawdust after 12 weeks insertion, down to 5.3×10^3 cells/g after 36 weeks may be due to either seasonal factors (the levels were determined before spring through to the end of summer), or to fungal succession and displacement of the bacterial population.

The highest estimated population of bacteria in CCA-treated wood in this study, 7.3×10^3 cells/g sawdust, may be compared with aerobic bacterial numbers in a fertile, virgin soil of between 5.2×10^6–1.4×10^8 cells/g soil (Braid, 1978).

A small number of non-cellulolytic creosote-tolerant bacteria were present in samples of creosote-treated E. obliqua stakes. The bacterial levels varied from 2.3×10^3 – 5.4×10^3 cells/g sawdust. Bacterial metabolism of creosote has been studied and reported previously by workers including Stranks and Hulme (1976) and Line (1977).

4.4 Development of Assay Techniques for the Assessment of Wood Degradation

The pH optimum for Cx-cellulase activity of microbial-degraded wood sawdust (Figure 3) was similar to those reported for a variety of culture filtrates of cellulolytic fungi (Trichoderma viride, Sternberg, 1976; Phoma hibernica, Urbanek, Zalewski-Sobczak and Boronwińska, 1978; Phialophora malorum, Berg, 1978). The optimum temperature for Cx-cellulase activity in sawdust was similar to those of pure cultures of several microorganisms (Aspergillus sp., unidentified Actinomycete, Reese, Siu and Levinson, 1950; Chaetomium thermophile var. dissitum, Eriksen and Goksøyr, 1976; unidentified Basidiomycete, Shewale and Sadana, 1978; Penicillium verruculosum, Szakács, Réczey, Hernadi and Dobozi, 1981). The sharp decline in enzyme activity at temperatures above 50-55°C (Figure 4) would be due to enzyme inactivation or denaturation (Goksøyr, Edisâ, Eriksen and Osmundsvåg, 1975).

Methods involving the alkaline degradation (deacetylation) of chitin have been used repeatedly in the estimation of fungal biomass in plant material (Ride and Drysdale, 1972; Hepper, 1977; Sharma, Fisher and Webster, 1977; Whipps, Clifford, Roderick and Lewis, 1980). The reported techniques necessitate the use of time-consuming procedures not amenable to large-scale application. The alkaline deacetylation technique was not sensitive compared with acid hydrolysis (Figure 6) and resulted in incomplete estimations of fungal chitin in plant tissues (Ride and Drysdale, 1972).

Acid hydrolysis of chitin has generally involved incubation periods of 2-3h at 95-110^{o}C for the decomposition of chitin to glucosamine, and the release of various amino-sugars from animal tissue (Boas, 1953; Swann and Balazs, 1966; Tsuji et al., 1969). Under these conditions, sealed flasks containing N_2 gas (Hubbard, Seitz and Mohr, 1979) were occasionally used to minimize destruction of glucosamine [the compound is largely destroyed by prolonged heating in strong acid at 100-110^{o}C (Boas, 1953; Dawson and Mopper, 1978)].

Hydrolysis of chitin involves a dynamic balance between liberation and destruction of glucosamine. The presently described technique employs a lower temperature (80^{o}C) and a longer hydrolysis time (20h) in air, thus simplifying the procedure. There appeared to be little destruction of glucosamine under these conditions (Figure 7). However, good recovery of glucosamine was dependent upon the condition of the ion-exchange columns (Plate 19) which show a rapid loss of efficiency when used repeatedly. Therefore, known quantities of purified chitin must be included in assays as controls for such losses.

Rotary evaporation of the hydrolysates has previously been applied to remove the acid after hydrolysis (Swift, 1973; Toppan, Esquerré-Tugayé and Touzé, 1976; Hubbard, Seitz and Mohr, 1979). However, this step was eliminated simply by diluting the hydrolysates and using sub-samples for subsequent ion-exchange chromatography.

Formerly, a variation of the Elson and Morgan (1933) technique has been generally employed for the colorimetric estimation of glucosamine. The basic method is not sensitive, however, and precise conditions are required for maximum expression of colour (Boas, 1953; Enghofer and Kress, 1979).

Sawicki, Hauser, Stanley and Elbert (1961) originally used MBTH for the detection of water-soluble aliphatic aldehydes but Tsuji et al. (1969) developed a procedure for estimating amino-sugars with the compound.

The amino-acid L-tryptophan produced a marked colour reaction with MBTH compared with the standard (Table 7). It is unlikely that L-tryptophan would interfere with glucosamine determinations, as it would be largely destroyed during acid hydrolysis of chitin (Robinson, 1963).

The low-level colour reaction of sound, freshly cut wood with MBTH reagent may be due to the existence of unknown compounds (Figure 10). It is possible though, that the reaction may be attributable to microorganisms present in living sapwood (Knutson, 1973; Bagley, Seidler, Talbot and Morrow, 1978; Shortle and Cowling, 1978b). Glucosamine is also found in bacteria (Sharon, 1965); presumably wood-inhabiting organisms would contribute to the levels of wood glucosamine.

Galactosamine, an amino-sugar similar in structure to glucosamine, yielded colour when reacted with MBTH (Tsuji et al., 1969). It is absent from living, sound wood and is not commonly found in large concentrations in imperfect fungi, apart from some Aspergillus spp. (Distler and Roseman, 1960; Sharon, 1965; Bartnicki-Garcia, 1968). It is unlikely that it would be of much significance in the assay.

The chitin contents of selected fungi vary both between species and with time of culture within one species [Table 9; Figure 11(ii)]. There is a general positive relationship between total fungal chitin content and total fungal weight within an individual species, although fungal chitin content as a proportion of the weight may vary depending on mycelial age [Figure 11(ii)].

The range of chitin contents between species of fungi examined (Table 9) was considerable. Blumenthal and Roseman (1957) found similar variations in chitin levels in 25 fungal strains examined. Thus, it would be expected that the chitin contents of different fungal species in wood would be equally variable.

Alterations in cultural conditions have been reported to result in fungal chitin content changes: fungal age, environment and morphology all contribute to different chitin levels (Sharma, Fisher and Webster, 1977). The observed decline in chitin content of Trichoderma viride [Figure 11(ii)] was presumably due to mycelial autolysis and chitinase activity in cultures whose exogenous energy sources have been largely depleted (Trinci and Righelato, 1970; Bartnicki and Lippmann, 1972; Smith and Berry, 1974).

The calibration or correction factor (Swift, 1973) between chitin contents and mycelial biomass estimates in plant tissue is of necessity calculated from chitin contents of fungi grown in vitro and hence is prone to error. However, Ruiz-Herrera (1978) determined the mean chitin contents of Deuteromycetes reported in the literature to be approximately 10% of dry mycelial weight, a figure similar to that derived in the present study. In any event, glucosamine concentrations of degraded woods assessed using acid hydrolysis followed by colorimetric estimation by MBTH, would give comparative information on the extent of fungal attack.

Good correlations were observed between Cx-cellulase assays, mycelial biomass estimates, fungal propagule numbers and microscopic estimates of cell-wall degradation, of radial transect samples through the ground-line of Eucalyptus sp. transmission poles (Figure 13). Different aspects of the fungal degradation process were examined by the individual methods, though. Fungal propagule counts provide a measure of the colony-forming units per unit of sawdust at the time of sampling, whilst the chitin assay appraised both present and probably a degree of past fungal growth. Since the Cx-cellulases have been found to be relatively stable in this study (Figure 19), and also by Enari and Markkanen (1977), and Reese and Mandels (1980), assay for this enzyme would also indicate both present and some past activity in wood samples.

Wood degradation can be estimated by sectioning and microscopic examination, but the technique was found to be time-consuming and largely subjective in this study. Accurate microscopic estimates require examination of a large number of representative sections due to the small area of each individual observation combined with variability of fungal attack.

A limitation of the fungal propagule count technique was demonstrated in field trial assessments. Where fungi were present in wood samples in the hyphal state, the number of propagules obtained per unit of sawdust would be expected to be proportional to the viable fungal biomass present. If, however, the fungi sporulated within wood cells as was occasionally observed, a cross-contamination of sterile sawdust particles was inevitable. This would result in excessively high and variable counts, bearing little relationship to the biomass.

It should also be stressed that fungal growth on cellulose agar does not necessarily indicate cellulolytic activity, since fungi have considerable capacity to grow in nutrient limited media (Wood, 1969; Nilsson, 1974b).

The Cx-cellulase assay provided an estimate of only one enzyme involved in the wood degradation process. A number of reports have been made of NaCMC-ase activity in fungi which cannot degrade native crystalline cellulose (Reese and Levinson, 1952; Wood, 1969; Nilsson, 1974b). Nonetheless, the presence of NaCMC-ase activity in timber is strongly indicative of cellulolytic fungal activity. The Cx-cellulases (endoglucanases) have been the central enzymes in all cellulolytic systems examined (Goksøyr, 1975) and the Cx-cellulase activity has been found to correlate well with the degree of soft-rot in the wood samples tested.

4.5 Field Trial Assessments of Assay Methods for Wood Degradation Estimation and Wood Preservative Performance

If biological techniques for the assessment of wood degradation, such as the Cx-cellulase assay and the fungal propagule count on cellulose agar, are to be used, then they must perform better than the Pilodyne(R) which has the considerable advantages of speed, non-destructive action and simplicity of operator interpretation. Such an improved performance was demonstrated for the enzyme assay (Table 10) when compared with the 6J Pilodyne. No great improvement in Pilodyne test precision through increased penetration (Friis-Hansen, 1980) into eucalypt hardwood, was gained by using a higher energy (10J) device with an increased pin diameter (Figure 14). The principle disadvantage of the Pilodyne observed in this examination, was the necessity of uniform wood moisture conditions, a fact previously noted by Hoffmeyer (1978).

Remedial wood preservatives at the Warrane (Tasmania) *Eucalyptus* sp. pole stub site were assessed by use of the enzyme assay and fungal propagule estimates on cellulose agar [Figures 15(i)-15(iv)]. Considerable variability existed between pole replicates of the same preservative treatment when assessed by both methods (Appendix 7). This effect would be due to the pocketing effect of soft-rot decay in wood poles.

An attempt was made to minimise this variability by increasing the number of drill cores obtained from two per pole stub [Sample (i), January-February, 1979 and Sample (ii), August, 1979] to five per stub [Sample (iii), February, 1981]. However, some variability persisted. It was considered by McPherson (1979, pers. comm.) that, in the original design, a minimum number of 10 stubs per treatment would have been required to markedly reduce this error.

A seasonal effect on the activity of wood pole-inhabiting fungi was apparent at the Warrane site. Control (untreated) stubs had higher levels of activity at August, 1979, than January, 1979, when measured by the Cx-cellulase assay and the fungal propagule count. The levels of activity then declined by 70-80% [Figures 15(i)-15(iv)] between August, 1979, and February, 1981. The February, 1981, sampling was preceded by a three-month period of high temperatures and moisture stress, factors contributing to the activity decline. These observations substantiate a report by Line (1979) who noted similar decreases in Cx-cellulase activity at the ground-line of <u>Eucalyptus</u> sp. poles following an extended period of dry weather. Earlier, Verrall (1939) recorded a similar effect of temperature on the growth of sap-staining fungi in wood and Theden (1972) has noted that some Basidiomycete fungi could not tolerate moderate temperatures ($27^{\circ}C$) for 6 months in dry wood.

Some preservative-containing bandages moderated this effect. Both the C.S.I.R.O.-developed Blue 7 and Busan 30 (Mark IV) cross-linked polyethylene bandages, and to a lesser degree, the Wolman bandage, protected the Cx-cellulases produced by microorganisms prior to bandage application. These treatments may have retarded the desiccation of the poles or insulated the woods from solar heat. Due to their inferior construction, the Tanalith C and copper napthenate bandages were not effective in this regard.

It is perhaps unlikely that the existence of the Cx-cellulase (endoglucanase, EC 3.2.1.4) component in the woods continues to contribute to gradual cellulose hydrolysis after fungal death. Both in this study (Figure 19) and in the report by Enari and Markkanen (1977) the high degree of endoglucanase stability has been noted. However, Enari and Markkanen (1977) also mentioned that exoglucanases were quite labile in comparison. It is difficult to perceive that cellulose breakdown would occur in timber in the presumed absence of an essential component of the cellulase complex.

Phialophora mutabilis and _Oidiodendron griseum_ were among the predominant soft-rot fungi isolated from Tasmanian pole species (Line and Cruickshank, 1979). Hence, the presence of these fungi in remedial-treated pole stubs at Warrane was considered to be strongly indicative of continuing soft-rot attack.

Comparing the candidate wood preservatives tested, the Busan 30, Blue 7, and Wolman bandages had excellent fungicidal effectiveness to 20mm depth and good penetration of the toxic components was observed in the time studied. This rapidity of diffusion was also noted by Da Costa and Collett (1979) for Busan 30, Blue 7 and a Wolman bandage preservative. Apart from the PCP bandage, the remaining preservatives appraised had fair to ineffectual levels of performance as tested. The bituminous paint application may have encouraged fungal survival and growth.

As a remedial wood preservative, creosote was unsatisfactory in this trial due to poor diffusion through the sapwood. The slow diffusion of PCP indicated by the narrow zone of fungicidal action [Sample (ii), Figure 15] has been previously reported by Da Costa and Collett (1979). However, its excellent fungicidal effectiveness has been noted by workers including Cockroft (1973), Scheffer and Eslyn (1978) and Johnstone, Gardner and Pitt (1979). In addition, the PCP bandage appeared to produce a denaturing effect on the Cx-cellulases present in the stub sapwoods.

A similar trend between the fungal propagule count on cellulose agar and the Cx-cellulase assay for samples from the Wedding Bells trial at Coff's Harbour, N.S.W., was observed (Figure 16). Unfortunately, samples from untreated control stubs were not procured for comparison of fungicidal effectiveness.

The suitability of Pinus radiata as a timber for use in ground contact (Savory, 1954b; Butcher, 1975) and the efficacy of pressure-impregnated high temperature creosote was indicated by estimates and activities in samples taken from the site. The CCA-treated Eucalyptus maculata stubs were more susceptible to soft-rot attack than the creosote-treated woods. Termite attack of the C.S.I.R.O. (Mark II) Blue 7 bandages may have contributed to their inferior performance when compared with the C.S.I.R.O. BFB bandages.

The preservative-containing C.S.I.R.O. Mark IV heat-shrink bandages had been applied only 12 months prior to the time of testing. A longer period would be needed before a reasonable assessment of the systems could be made. However, results from tests of both the Warrane (Tasmania) and Coff's Harbour (N.S.W.) trials indicate considerable promise for these bandages as remedial and ground-line maintenance treatments.

Analysis of the Eucalyptus obliqua sapwood stakes positioned at Grove, Tasmania, allowed further evaluations to be made of assay methods and wood preservative treatments.

The fertile soil conditions at the site (Appendix 11) coupled with the high annual rainfall, ensured rapid microbial attack of the wood.

With the exception of creosote-treated stakes, the high levels of Cx-cellulase activity demonstrated in all treated and untreated woods after 36 weeks field insertion was probably the result of cellulase production by microorganisms. Although the existence of endogenous wall-degrading enzymes (including cellulases) has been reported (Sassen, 1965; Lee, Kivalaan and Bandurski, 1967; Karr and Albersheim, 1970; Kozlowski, 1973), no such Cx-cellulases were found in freshly cut eucalypt sapwood in this investigation. The Cx-cellulase activities of creosote-treated stakes were probably artificially high as propylene oxide (used as a sterilizing agent) solubilized the fixed creosote and raised the control viscosity.

A flaw in the fungal propagule count technique for estimation of relative viable mass per unit of wood in treated sapwood was apparent in this investigation. Trichoderma viride over-ran cellulose agar plates containing sawdusts from Tanalith C and untreated (control) sapwood inserted at the Grove site. Fungal biomass in wood would have been underestimated in these treatments. Curously, T. viride was not isolated from boric-acid treated timber even though the preservative was a comparative failure (Appendix 9).

Wide variations in mycelial biomass estimates (obtained from assay of chitin contents) were noted for the wood treatments (Figure 17) with the untreated control and boric-acid-treated stakes producing the highest microbial attack. This corresponded well with the Cx-cellulase activities and relatively high fungal propagule counts recorded for the two woods. Low fungal biomass levels for creosote, NaPCP, and $CuSO_4/K_2CrO_4$-treated woods also correlated well with the respective enzyme and fungal propagule estimates.

Trichoderma viride was the predominant organism isolated from Tanalith C-treated sapwood after ground emplacement. As noted previously, this fungus is a potent producer of Cx-cellulases in culture. Although contributing little to fungal biomass estimates (Figure 17), the organism may have produced high levels of Cx-cellulase activity in the wood. Alternatively, the low apparent biomass estimates (from chitin assays) may be due to the high concentrations of CCA-preservative observed on the outer wood surface, exerting an oxidizing effect on glucosamine during acid hydrolysis. These high CCA concentrations would have to be considerably higher than those tested previously for such oxidative effects (Table 8).

Application of the Least Significant Difference (L.S.D.) statistical test to the results of the Grove stake trial showed creosote to be the best performed preservative [Figure 18(i)], whilst the untreated control and boric acid-treated woods had high levels of microbial activity [Figure 18(ii)] when assessed by all methods and compared with creosote. As the remaining preservatives had varying levels of performance according to the test method employed, it is apparent that when inspection of timber for incipient decay is required, a combination of analytical test procedures should be used. Different information on the same degradative process is provided by each technique examined in this study.

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APPENDICES

Appendix 1

Composition of Spurr's (1969) and Araldite embedding media:-

(i) Spurr's resin. The composition was:

vinylcyclohexene dioxide (V.C.D.)	5.0g
diglycidyl ether of polypropylene glycol (D.E.R.)	3.0g
nonenyl succinic anhydride (N.S.A.)	13.0g
dimethyl amino ethanol (D.E.A.E.)	0.1g

All above reagents were supplied by Ladd Chemicals, Burlington, U.S.A.

(ii) Araldite resin. The composition was:

Araldite M	37.3g
Araldite HY964	27.9g
Araldite DY064	0.48g
di-n-butyl phthalate	1.56g

Di-n-butyl phthalate was supplied by B.D.H. Chemicals, Poole, England. The remaining Araldite components were supplied by CIBA-Geigy, Lane Cove, Australia.

Appendix 2

Sampling localities of CCA-treated *Eucalyptus* sp. poles for:

1. Studies into the relationships between fungal propagule and bacterial counts from wood samples (Fig. 2).

2. Isolation of bacteria for enzymic studies with wood substrates (Table 5-6).

a. Northern Tasmanian sample localities

Pole No.

1. High Street, Sheffield
2. Nook Road (via Sheffield)
3. Shorey's Road, near West Kentish
4. Valley Road, Devonport
5. The Bluff, Devonport
6. Sassafrass
7. Weston Street, Deloraine
8. Drummond Street, Perth
9. Main Street, Campbell Town
10. Barrack Street, Oatlands

b. Southern Tasmanian sample localities

Pole No.

1. Heathcote Avenue, Sandy Bay, Hobart
2. Seaview Avenue, Taroona, Hobart
3. Taronga Road, Hobart
4. Margate Road, Kingston
5. Main Road, Margate
6. Tinderbox Road, Howden
7. The Esplanade, Blackmans Bay
8. The Esplanade, Kingston Beach
9. French Street, Dynnyrne
10. Huon Road, South Hobart

The notation used in Table 5 for bacterial identification described the following:

S 6 S A

Sample series

pole 6

southern pole aspect

1st isolate chosen from that core sample after plating on TYE

N 6 N B

Northern sample

pole 6

Northern pole aspect

second isolate randomly chosen

Appendix 3

Weight loss of *Eucalyptus obliqua* sapwood blocks exposed to fungi isolated from Tasmanian CCA-treated transmission poles: Data for Table 4.

	Experiment 1			Experiment 2		
Fungal Isolate	Block No.	Percent weight loss	Corrected percent weight loss	Block No.	Percent weight loss	Corrected percent weight loss
Alternaria sp.	34	10.63	0.35	21	9.01	3.73
	59	10.56	0.28	7	8.47	3.19
	63	11.73	1.45	48	8.93	3.65
	25	11.38	1.10	27	11.23	5.95
Aureobasidium pullulans	15	11.45	1.17	3	5.52	- 0.24
	13	9.47	0.00	53	6.25	0.97
	41	10.50	0.22	19	5.41	0.13
	17	9.83	0.00	35	8.11	2.83
Chaetomium globosum	NT	NT	NT	36	6.40	1.12
				60	8.00	2.72
				39	7.15	1.87
				61	5.97	0.69
Doratomyces microsporus	35	15.82	5.54	29	10.12	4.84
	10	11.49	1.21	5	9.57	4.29
	26	11.59	1.31	13	9.54	4.26
	3	10.75	0.47	37	10.05	4.77
Fusarium decemcellulare	46	10.01	0.00	9	6.28	1.00
	12	11.56	1.28	58	6.57	1.29
	55	12.04	1.76	22	7.99	2.71
	37	10.95	0.67	33	6.37	1.09
Graphium rigidum	33	15.22	4.94	2	9.32	4.04
	21	15.26	4.98	17	9.31	4.03
	7	13.62	3.34	46	7.35	2.07
	24	12.44	2.16	28	12.47	7.19
Oidiodendron griseum	38	10.06	0.00	15	7.56	2.28
	29	12.06	1.78	47	7.82	2.54
	16	10.26	0.00	10	6.51	1.23
	32	10.76	0.48	26	7.07	1.79
Paecilomyces varioti	9	10.97	0.69	30	11.70	5.72
	58	10.51	0.23	62	10.27	4.99
	23	10.68	0.40	66	5.56	0.28
	50	10.63	0.35	16	5.45	0.17
Penicillium frequentans	14	10.89	0.61	NT	NT	NT
	43	10.71	0.43			
	49	10.68	0.40			
	36	10.63	0.35			
Phialophora mutabilis	54	10.63	0.35	43	9.41	4.13
	47	15.32	5.04	54	8.15	2.87
	11	10.48	0.20	52	6.40	1.12
	42	14.83	4.55	51	11.46	6.18
*Polystictus versicolor**	NT	NT	NT	59	43.91	38.63
				67	43.17	37.89
				11	34.16	28.88
				41	19.62	14.34
Pycnostanus sp.	48	12.28	2.00	NT	NT	NT
	57	12.48	2.20			
	31	11.57	1.29			
	5	13.75	3.47			

Appendix 3 (continued)

Fungal Isolate	Experiment 1 Block No.	Percent weight loss	Corrected percent weight loss	Experiment 2 Block No.	Percent weight loss	Corrected percent weight loss
Pyrenochaeta sp.	1	17.18	6.90	55	8.14	2.86
	2	11.87	1.59	65	14.07	8.79
	56	12.11	1.83	24	11.81	6.53
	30	11.76	1.48	44	9.70	4.42
Trichoderma viride	51	10.94	0.66	32	6.40	1.12
	20	9.78	0.00	69	6.58	1.30
	22	10.81	0.53	64	5.84	0.56
	39	11.73	1.45	45	6.24	0.96

*White-rotting Basidiomycete was included for comparison.

Experiment 1 - Blocks measured 20 x 20 x 20mm.

Experiment 2 - Blocks measured 30 x 20 x 10mm.

Twelve control (uninoculated) blocks (3 flasks, 4 blocks per flask) were incubated in parallel with the inoculated sapwoods. The mean weight losses of the 12 blocks in both experiments were:-

Experiment 1 - 10.28%

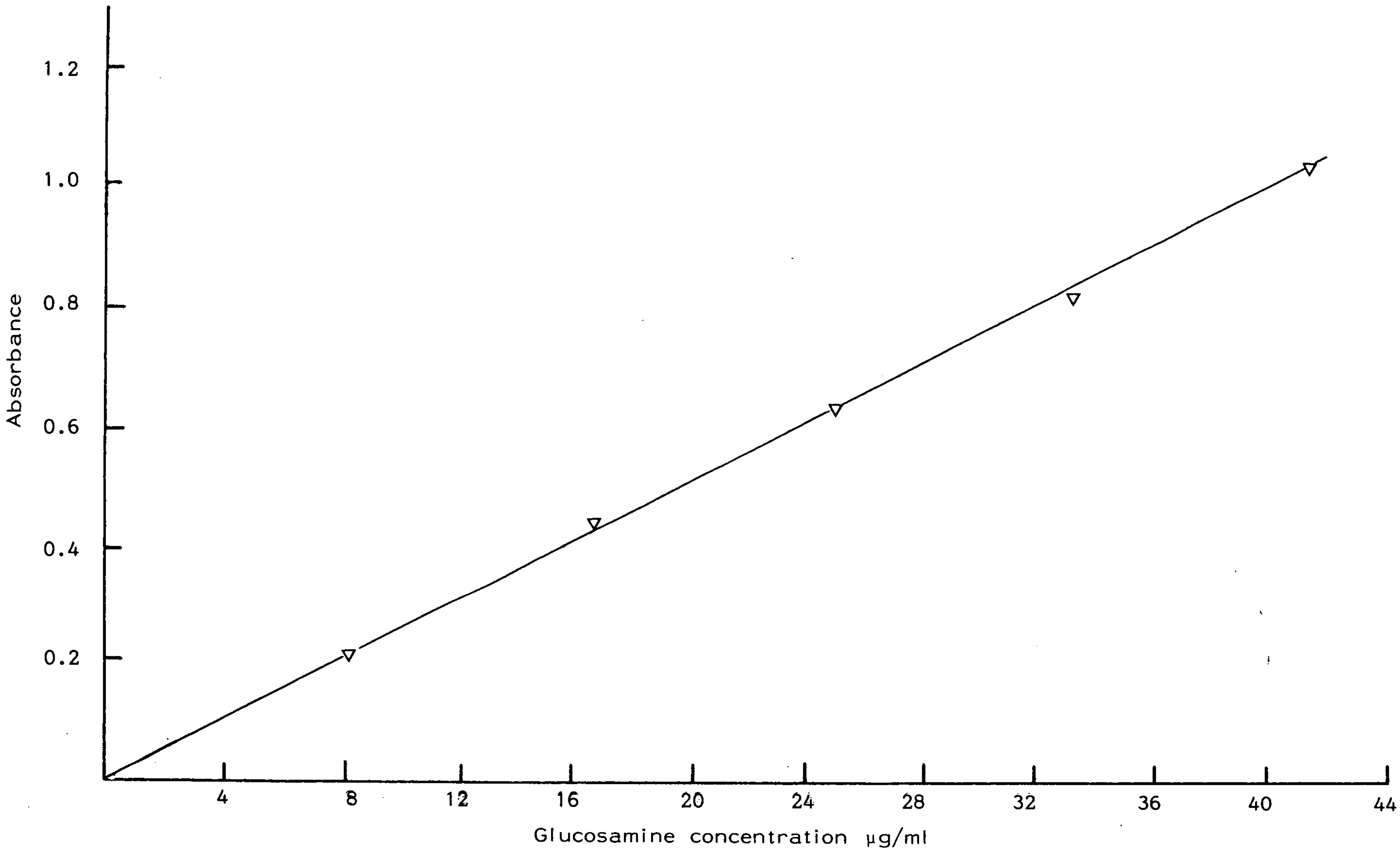
Experiment 2 - 5.28%

The corrected sapwood weight loss was obtained by subtracting the respective mean control weight loss from the individual inoculated block weight losses.

The incubation time was 16 weeks at 22°C.

Appendix 4

Standard curve of glucosamine + MBTH reagent against reagent blank at 630m μ. Each point is the mean of duplicate determinations.


1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
4
8
12
16
20
24
28
32
36
40
44
Glucosamine concentration µg/ml

Appendix 5

Grafton, N.S.W., Pole Stub Trial - data for Table 10.

Treatment	Stub No.	Percent degradation of 0.4% NaCMC	Mean Pilodyne(R) penetration (mm)	Predominant fungi present
Untreated with shrinkwrap sleeve	1	27.8	23.7	P
Untreated with shrinkwrap tape	2	25.0	19.3	P + Pen + Pm + T
CCA controls	43	4.3	8.0	P + Pen
	44	8.9	10.3	P + Pen + Pm
	47	4.8	11.5	P + Pen + T + *Alternaria*
	50	10.9	10.5	P + Pen + Pm
	52	2.2	10.8	P + Pm + T
CCA + shrinkwrap sleeve	3	1.5	12.0	P
CCA + shrinkwrap tape	4	2.2	10.5	P + T
CCA + tape X, no primer, 1 wrap	7	1.0	11.5	P + Pen + *Sporothrix*
2 wraps, no primer	16	5.8	10.3	P + Pen + T
2 wraps + primer	9	<1.0	10.0	P + Pen
1 wrap + primer	5	1.5	9.0	Pen + P
CCA + tape Y, no primer, 1 wrap	8	1.5	9.8	P
2 wraps, no primer	17	<1.0	12.0	P + Pen
2 wraps + primer	15	1.5	8.8	P + Pen
1 wrap + primer	6	<1.0	11.5	P + Pen
CCA + oxy-char	10	2.2	13.0	P + T
CCA + Char + PCP	(?11)12	<1.0	10.5	P
CCA wettex creosote	18	0.0	11.5	P + Pen
CCA H.D. creosote	19	0.0	12.3	P + *Cladosporium* + *Epicoccum*
CCA + PCP + creosote	20	0.5	9.8	P + Pen
CCA + 3M flexible sealer	21	<1.0	8.5	P + Pen
CCA + Biersdorf tape	46	0.0	9.8	P + Pen
CCA + Biersdorf vulcanizing tape	48	2.6	10.8	P + Pen
CCA + KAP enamel Co	49	<1.0	12.5	P
CCA + KAP enamel HO	54	1.6	9.3	P + Pen

Appendix 5 (continued)

Eucalyptus maculata pole stubs (200-300mm diameter) were inserted at the site. The time interval between emplacement and sampling was 2 years, 2 months.

Enzyme (Cx-cellulase) assay data and fungal identifications were supplied by Dr. M.A. Line.

Abbreviations used: P = *Paecilomyces varioti*

Pen = *Penicillium* spp.

Pm = *Phialophora mutabilis*

T = *Trichoderma viride*

Enzyme assay values were means of duplicate determinations. Sawdusted wood samples (0.3g) were incubated in 10ml 0.4% NaCMC in 0.1M acetate buffer for 1h at 45°C.

Pilodyne(R) penetration readings were means of 4 readings per pole stub at the ground-line. (R) = Registered trademark.

The predominant fungi in the wood samples were determined using 0.25% swollen cellulose agar, 0.01g sawdust per plate. The plates were incubated for 12d at 22° C before inspection.

Appendix 6

Regression of the pin penetration of a 6J, 2.0mm pin diameter Pilodyne(R) on the pin penetration of a 10J, 2.5mm pin diameter Pilodyne(R): Data for Figure 14.

Stub No.		Pilodyne(R) Pin Penetration (mm)	
		6J, 2.0mm pin diameter	10J, 2.5mm pin diameter
112	S	14, 14	20, 14
	N	13, 14	17, 18
111	S	8, 8	17, 18
	N	12, 13	13, 17
110	S	14, 13	18, 14
	N	14, 16	18, 16
109	S	8, 10	10, 8
	N	8, 7	12, 11
108	S	10, 10	12, 16
	N	10, 10	11, 10
107	S	9, 9	11, 11
	N	8, 10	10, 11
106	S	9, 11	12, 14
	N	10, 11	14, 13
105	S	9, 10	12, 12
	N	9, 12	10, 10
104	S	9, 10	12, 14
	N	10, 11	12, 14
103	S	10, 10	12, 11
	N	10, 9	12, 9
102	S	13, 12	14, 13
	N	10, 11	13, 12
96	S	9, 8	10, 10
	N	11, 10	20, 12
95	S	11, 11	11, 8
	N	9, 11	10, 10
94	S	8, 8	8, 8
	N	7, 8	10, 11
93	S	8, 8	10, 10
	N	9, 9	12, 9
92	S	8, 9	10, 12
	N	8, 9	12, 12
91	S	12, 9	11, 12
	N	8, 10	14, 12

Appendix 6 (continued)

S = Southern stub aspect

N = Northern stub aspect

Eucalyptus sp. wood stubs, cut from CCA-treated transmission poles and emplaced at Warrane, Tasmania, for one year, were tested at the ground-line.

(R) = Registered trademark.

Appendix 7

The Warrane (Tasmania) Pole Stub Trial.

Trial Plan

15m

22.5m

N

31	41	51	61	71	81	
32	42	52	62	72	82	
33	43	53	63	73	83	
34	44	54	64	74	84	
35	45	55	65	75	85	
36	46	56	66	76	86	97
37	47	57	67	77	87	98
38	48	58	68	78	88	99
39	49	59	69	79	89	100
40	50	60	70	80	90	101

Stub nos. 1-30, 37, 46, 55, 64, 85 and 91-96 were not examined in this study.

The *Eucalyptus* sp. pole stubs were 300-350mm in diameter.

Preservative treatments were applied to stub nos. 31, 41, 83, 89, 90 (Tanalith "C" bandage); 32, 43, 52, 61, 80 (Wolman bandage); 33, 42, 51, 81, 82 (copper naphthenate backfill); 34, 70, 73, 79, 88 (bituminous paint); 35, 44, 53, 62, 71 (Blue 7 bandage); 36, 45, 54, 63, 72 (Busan 30 bandage); 38, 47, 56, 65, 74 (PCP bandage); 39, 60, 69, 78, 87 (copper napthenate bandage); 40, 49, 58, 67, 76 (creosote paint); 48, 57, 66, 75, 84 (creosote backfill); 50, 59, 68, 77, 86 (creosote bandage), 97-101 (controls, untreated stubs).

Preservative formulations and methods of application

(a) Toxicant-containing bandages

i. 20% copper naphthenate (Koppers Aust. Pty. Ltd.) in white spirits. Approximately 1.5kg mix was applied to each pole stub at the ground-line. Toxicant support was hessian, 4 layers per stub, covered with 0.25mm clear polyethylene plastic.

ii. Creosote (High Temperature, HT - AS1143/73, Pabco-Koppers Pty. Ltd., Sydney). Approximately 1.5kg was applied to each stub. Toxicant support was 4 layers hessian applied to each stub, covered with 0.25mm clear polyethylene plastic.

iii. C.S.I.R.O.-developed bandage systems. Both Busan 30 and Blue 7 bandages were C.S.I.R.O. Division of Building Research Mark IV cross-linked polyethylene (XLPE) heat-shrink toxicant supports. The Busan 30 bandage contained 30% 2-(thiocyanomethylthio)benzothiazole (Buckman Labs., Memphis, U.S.A.) active ingredient. The Blue 7 bandage contained a patented copper/fluorine/boron preservative developed by CSIRO/DBR.

iv. 15% Tanalith C solution (Koppers Aust. Pty. Ltd.). The approximate salt concentration was: $K_2Cr_2O_7$, 45%; $CuSO_4.5H_2O$, 35%, $As_2O_5.2H_2O$, 20%. Two-three kg of preservative was applied to each stub. The toxicant support was 4 layers of hessian (pole stub nos. 31, 41, 83) or polyurethane foam (pole stub nos. 89, 90). The polyurethane foam bandages had 0.5mm black PVC for protection, whilst the hessian bandages were covered with 0.25mm clear polyethylene plastic.

v. 5% pentachlorophenol (PCP) in Caltex 55 process oil. Approximately 2kg of mix was applied to each stub. The toxicant support was 4 layers of hessian covered with 0.25mm clear polyethylene plastic.

vi. Wolmanol CFB bandage (Koppers Aust. Pty. Ltd.). The patented preservative system was copper/fluorine/boron-based.

(b) <u>Paints</u>

i. HT creosote, brush applied.

ii. Bituminous paint (Pabco, Sydney, Aust.), brush applied.

(c) <u>Soil backfills</u>

i. HT creosote, 30L 'puddled' in soil around the base of each pole stub.

ii. Copper naphthenate (20% in white spirits), 30L 'puddled' in soil around the base of each pole stub.

Mr. A.D. Shaw and Mr. G. McKinlay of the Hydro-Electric Commission of Tasmania devised bandages for copper naphthenate, creosote, Tanalith C and PCP preservatives. All preservatives were applied by the H.E.C. except the CSIRO-developed bandage systems, which were applied by Mr. C.W. Chin and Mr. C. McEvoy of CSIRO Division of Building Research.

Appendix 7 (continued)

Cx-cellulase and fungal propagule count – data for Figures 15(i)-(iv).

Sample (i) - January-February 1979

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant Fungi
51	Copper naphthenate backfill	24.6	6.9	Phial.
42		4.4	10.0	Phial., Oidio., Pen.
33		4.9	3.8	Paec., Phial., Alt.
81		0.5	0.3	Phial.
82		6.7	13.5	Pen., Phial.
61	Wolman CFB bandage	19.2	4.5	Phial., Pen.
52		26.5	28.0	Phial., Alt., Fus., Pen.
43		2.8	7.5	Pen., Phial., Fus.
80		1.9	5.0	Phial.
32		3.5	19.0	Phial., Pen., Fus.
71	Blue 7 bandage	5.0	9.0	Paec., Phial., Oidio., Pen.
62		6.4	0.3	Alt.
53		5.3	2.8	Phial., Alt., Pen.
44		8.7	6.0	Fus., Pen.
35		3.7	15.0	Pen., Phial., Fus.
72	Busan 30 bandage	1.1	6.5	Phial.
63		1.3	0.5	Oidio., Fus.
54		7.6	4.0	Phial., Alt., Fus., Cand.
45		6.1	6.5	Paec., Pen., Fus., Phial.
36		1.8	3.5	Phial.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant Fungi
73	Bituminous paint	2.7	1.5	Pen.
34		1.2	4.0	Phial., Fus.
88		6.8	0.3	Phial.
79		0.6	6.7	Pen., Phial.
70		3.2	33.0	Pen.
74	PCP bandage	33.7	48.0	Phial.
65		3.0	0.3	Pen.
56		0.0	1.0	Oidio., Fus., Phial.
47		2.5	2.8	Oidio., Pen., Paec.
38		4.1	7.3	Pen., Paec., Alt.
84	Creosote backfill	6.9	3.8	Fus., Phial., Cand.
75		3.2	1.0	Phial.
66		3.7	2.8	Pen., Phial., Paec.
57		0.0	3.0	Pen., Cand., Phial.
48		10.8	4.5	Pen., Paec., Phial.
76	Creosote paint	5.6	0.8	Phial., Oidio.
67		2.7	22.5	Pen., Fus., Phial.
58		0.3	0.8	Cand., Pen., Phial.
49		6.6	2.0	Pen., Phial., Oidio., Fus.
40		6.8	9.5	Phial., Pen.
86	Creosote bandage	2.4	0.3	Pen.
77		6.3	3.7	Phial., Pen.
68		6.7	14.8	Phial., Pen.
59		0.9	3.3	Pen., Alt.
50		4.8	3.0	Phial.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
87	Copper naphthenate bandage	4.5	1.0	Trich., Alt., Pen.
78		10.6	15.5	Phial.
69		4.2	30.3	Pen.
60		2.1	0.5	Pen., Alt.
39		3.9	35.5	Phial., Pen., Oidio.
31	Tanalith "C" bandage	4.4	1.0	Pen., Oidio.
89		5.0	1.0	Pen., Phial.
41		8.3	3.5	Phial., Pen.
83		5.0	3.5	Alt., Phial., Pen.
90		4.4	4.3	Oidio., Phial., Alt., Pen., Fus.
97	Control	4.4	1.5	Pen.
98		3.7	24.3	Pen., Phial.
99		3.4	4.5	Phial., Pen., Fus.
100		3.9	5.5	Cand., Pen., Alt.
101		4.4	33.0	Pen., Phial.

Sample (ii) - August 1979

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
51	Copper naphthenate backfill	6.7	22.9	Pen., Cand.
42		5.0	13.0	Pen.
33		6.4	11.0	Cand., Pen.
81		8.6	2.3	Pen.
82		4.2	18.3	Pen., yeasts
61	*Wolman CFB bandage	1.5	43.8	Oidio., Pen., Phial.
52		14.2	16.8	Pen., Phial., yeasts
43		5.1	7.5	Pen., Phial., yeasts
80		6.3	37.8	Pen.
32		1.5	34.5	Pen., yeasts
71	Blue 7 bandage	2.5	1.8	Pen.
62		1.7	1.8	Pen.
53		9.8	0.0	
44		1.4	0.3	Clad.
35		7.0	0.5	Pen.
72	Busan 30 bandage	1.4	1.3	Pen., Phial.
63		4.5	0.8	Pen.
54		15.2	0.0	
45		0.9	0.0	
36		9.1	2.0	Pen.
73	Bituminous paint	5.1	29.3	Pen.
34		7.1	22.8	Pen.
88		5.5	34.8	Pen.
79		2.7	32.0	Pen. Asp.
70		0.0	60.0	Pen., Fus.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
74	PCP bandage	7.2	18.5	Pen.
65		3.0	13.0	Fus., Oidio., Pen.
56		1.7	27.5	Pen.
47		8.6	88.0	Pen., Alt., Fus.
38		1.0	43.0	Pen., Phial., Oidio., Pen.
84	Creosote backfill	4.7	7.8	Pen.
75		9.1	7.3	Fus.
66		1.7	11.0	Fus.
57		0.0	11.0	Asp., Paec., Pen.
48		44.0	35.3	Pen.
76	Creosote paint	3.2	5.8	Pen., Fus.
67		6.8	42.0	Pen. Fus.
58		5.6	67.3	Pen., Fus.
49		16.6	45.8	Pen., Fus., Paec.
40		2.2	38.5	Pen.
86	Creosote bandage	3.7	1.5	Paec., Pen.
77		2.2	6.8	Fus., Pen.
68		7.3	63.5	Pen., Fus.
59		1.0	51.5	Pen., Fus.
50		15.0	25.8	Pen., Alt.
87	Copper naphthenate bandage	0.8	9.5	Pen.
78		0.0	26.3	Pen.
69		3.2	46.0	Pen., Alt.
60		0.5	11.0	Pen., Phial.
39		4.0	3.3	Pen.
31	Tanalith "C" bandage	12.4	24.5	Pen., Cand.
89		1.8	27.0	Pen.
41		5.2	4.3	Pen., Alt., Gliocladium sp.
83		4.0	0.0	-
90		7.2	30.0	Pen., Paec.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
97	Control	2.2	38.0	Pen.
98		6.0	56.5	Pen., Alt., Cand.
99		8.3	38.5	Pen.
100		12.0	34.5	Pen., *Mucor* sp.
101		10.7	32.0	Pen.

Sample (iii) - January-February 1981

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
51	Copper naphthenate backfill	2.2	2.4	Pen., Phial., Oidio., Alt., Clad., yeasts
42		1.1	2.4	Fus., Phial., Clad., Ulo., Trich., Oidio.
33		8.7	2.6	Phial., Pen., Ceph., Clad.
81		3.3	2.6	Oidio., Trich., Ceph., Pen., *Phoma* sp.
82		5.9	3.0	Ceph., Oidio., Alt., Clad.
61	Wolman CFB bandage	0.2	0.2	Pen.
52		5.6	0.6	Pen.
43		0.0	0.0	-
80		7.7	0.2	Pen.
32		4.3	0.0	-

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
71	Blue 7 bandage	5.1	0.8	*Monilinia* sp., Fus., Oidio., Pyren.
62		3.7	0.0	-
53		10.0	0.0	-
44		2.2	0.8	Clad., Alt.
35		3.9	1.4	Fus., Phial., *Phoma* sp., Pen.
72	Busan 30 bandage	4.7	1.2	Oidio., Pen., *Myrothecium* sp.
63		5.2	1.4	Oidio., Pen.
54		12.0	0.6	Oidio.
45		2.8	0.0	-
36		7.9	0.2	Pen.
73	Bituminous paint	2.1	18.8	Pen., Oidio., Clad., Paec.
34		3.0	8.8	Pen., Vert., Phial., Alt., Ceph., Fus.
88		16.0	45.2	Oidio., Pen., Paec., Phial., Clad.
79		8.0	18.6	Pen., Phial., Oidio., yeasts, Trich.
70		6.0	13.0	Oidio., Pen., Fus., Ulo., Phial.
74	PCP bandage	2.8	3.2	Paec., Pen., Oidio., *Gliomastix* sp.
65		0.0	2.8	Clad., Oidio., Pen., Alt., Fus., *Chloridium* sp.
56		0.6	1.6	Clad., Ulo.
47		0.9	0.8	Pen., Ceph.
38		0.1	1.4	Pen., Clad., Alt., *Gonytrichum* sp.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
84	Creosote backfill	3.6	1.2	Pen., *Gliocladium* sp.
75		2.9	0.6	Clad., Phial., Fus.
66		10.3	12.0	Paec., Pen., Alt., Clad.
57		3.0	1.4	Pen., Phial., Oidio.
48		6.0	1.0	Pen., Clad., Fus.
76	Creosote paint	1.7	4.2	Ceph., Pen., Oidio.
67		3.5	6.2	Oidio., Fus., Pen., Alt., Amblyo.
58		2.1	3.4	Clad., Oidio., Pen., *Gliocladium* sp.
49		8.6	2.8	Trich., Clad., Oidio., Pen.
40		0.1	1.8	Pen., Fus., Oidio.
86	Creosote bandage	4.2	5.4	Clad., *Gliocladium* sp., Pen., Phial.
77		1.9	0.6	Ceph., Pen., Fus.
68		1.1	1.0	Ceph., Pen., Fus.
59		1.0	1.6	Pen., Alt., Fus.
50		3.9	1.4	Phial., Pen.
87	Copper naphthenate bandage	2.2	5.2	Pen., Phial., Oidio., Amblyo., Pyren.
78		1.7	3.0	Phial., Pen., Fus.
69		0.5	1.2	Fus., Clad., Ulo., Pen.
60		3.5	4.4	Pen., Oidio., Ceph., Phial.
39		1.6	0.4	Oidio., Clad., *Arthrobotrys* sp.

Stub No.	Treatment	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi
31	Tanalith "C" bandage	0.6	12.8	Clad., Pen., Fus., Alt., *Phoma* sp.
89		5.8	3.8	Pen., Oidio.
41		0.0	19.0	Oidio., Fus., Alt., Pyren., Pen.
83		0.2	4.2	Paec., Pen., Clad.
90		1.8	53.0	Pen., Fus., Clad., Phial., Oidio.
97	Control	0.0	1.6	Pen., Fus., *Doratomyces* sp., Phial.
98		3.0	7.4	Pen., Clad., Trich., *Umbelopsis* sp.
99		0.2	16.2	Oidio., Phial., Pen., *Aureobasidium* sp.
100		5.1	5.0	*Doratomyces* sp., Phial., Pen., Fus., Clad.
101		2.0	0.8	Oidio., Pen., Clad.

Abbreviations:

Alt. = Alternaria sp(p).

Amblyo. = Amblyosporium sp.

Asp. = Aspergillus sp(p).

Cand. = Candida sp(p). - yeast(s)

Ceph. = Cephalosporium acremonium

Clad. = Cladosporium herbarum

Fus. = Fusarium sp.

Oidio. = Oidiodendron griseum

Paec. = Paecilomyces varioti

Pen. = Pencillium spp.

Phial. = Phialophora mutabilis

Pyren. = Pyrenochaeta sp.

Trich. = Trichoderma viride

Ulo. = Ulocladium sp.

Vert. = Verticillium sp.

Enzyme assay (Cx-cellulase assay) values were the means of duplicate samples [Samples (i) and (ii)] or 5 replicate samples [Sample (iii)]. Sawdust samples (0.2g) were incubated in 10ml 0.4% NaCMC in 0.1M acetate buffer (pH 5.5) for 1h at 45°C.

Estimates of fungal propagule numbers per 0.01g sawdust on 0.25% swollen cellulose agar were means of 4 replicate plates (2 per core) [Samples (i) and (ii)] or 5 replicate plates (1 per core) [Sample (iii)]. After addition of sawdust, the plates were incubated at 22°C for 10d prior to inspection.

*Tested 5 months after other treatments [Sample (ii)] prior to preservative application.

Warrane Pole Stub Site

Enzyme (Cx-cellulase) Assay Results -

Analysis of Variance [Sample (iii) - January-February, 1981 only]

Source of Variation	df	SS	MS	F
Among poles	59	3032.1		
Treatments	11	936.2	85.1	1.9 ($p < 0.1$)
Among poles within treatments	48	2095.9	43.7	2.0 ($p < 0.001$)
Among samples within poles	240	5124.8	21.4	
Total	299	8156.9		

L.S.D. (0.05) = 3.8% reduction in viscosity NaCMC.

L.S.D. (0.01) = 5.1% reduction in viscosity NaCMC.

Warrane Pole Stub Site

Fungal propagule count data - 0.25% cellulose agar

Analysis of Variance [Sample (iii) - January-February, 1981, only]

Source of Variation	df	SS	MS	F
Among poles	59	27062.6		
Treatments	11	13115.6	1192.3	4.1 ($p < 0.001$)
Among poles within treatments (experimental) error)	48	13947.0	290.6	3.2 ($p < 0.001$)
Among samples within poles (sampling error)	240	21708.8	90.5	
Total	299	48771.4		

L.S.D. (0.05) = 9.7 fungal colony counts.

L.S.D. (0.01) = 12.8 fungal colony counts.

The above statistical analyses were performed according to Steel and Torrie (1960), page 121.

Appendix 8

Coff's Harbour, N.S.W., Pole Stub Trial - Data for Figure 16.

Treatment		Stub No.	Cx-cellulase assay - % reduction viscosity NaCMC	Fungal propagule count/ 0.01g sawdust	Predominant fungi (on 0.25% cellulose agar)
Pinus radiata - CCA	Blue 7	3B1	9.0	21	Phial.
		3B2	6.3	0	Phial.
		3B3	2.1	8	Aureo., Pen., Paec., Fus.
	BFB	3N1	2.4	7	Phial., Aureo.
		3N2	3.5	16	Phial., Clad.
		3N3	2.4	1	Pen.
Eucalyptus maculata - CCA	Blue 7	4B1	32.6	72	Phial., Paec., Clad., Pen.
		4B2	15.3	64	Phial., Pen.
		4B3	36.1	46	Phial., Pen.
	BFB	4N1	27.8	81	Phial., Pen., Gony.
		4N2	12.5	37	Paec., Pen., Fus.
		4N3	4.2	5	Pen., Paec., Phial.
Eucalyptus maculata - Creosote	Blue 7	5B1	5.9	0	-
		5B2	8.7	0	-
		5B3	2.1	0	-
	BFB	5N1	6.3	0	-
		5N2	NT	0	-
		5N3	6.2	0	-
Eucalyptus maculata - Untreated BFB (XLPE)		30-2x	6.9	0	-
		10-2x	0.0	0	-
		43-2x	19.8	0	-
		16-2x	0.0	1	Phial.
Eucalyptus maculata - CCA BFB (XLPE)		22x-4x	7.3	0	-
		4-4x	2.1	0	-
		14-4x	5.2	1	Phial.
		38-4x	3.5	15	Pen., Amb.

The Eucalyptus maculata-XLPE (Mark IV) bandage treatments were in position for one year only prior to testing. The remaining stubs plus treatments were inserted three years before examination. The pole stubs were 270-320mm in diameter.

<u>Appendix 8</u> **(continued)**

Abbreviations used:

Amb. - *Ambylosporium* sp.

Aureo. - *Aureobasidium pullulans*

Clad. - *Cladosporium herbarum*

Fus. - *Fusarium* spp.

Gony. - *Gonytrichum* sp.

Paec. - *Paecilomyces varioti*

Pen. - *Penicillium* spp.

Phial. - *Phialophora mutabilis*

Fungal propagule numbers were obtained from 0.25% swollen cellulose agar plates, 0.01g sawdust per plate.

The incubation period before counting was 12d at 22°C.

Cx-cellulase assay values were obtained from 0.3g sawdust samples incubated in 10ml of 0.4% NaCMC (pH 5.5) for 1h at 50°C.

Appendix 9

Grove (Tasmania) Stake Trial

Treatment A - HT creosote (Pabco-Koppers Aust. Pty. Ltd., Sydney, Aust.), hot and cold bath

B - Tanalith "C" Paste (Koppers Aust. Pty. Ltd., Sydney, Aust.)

C - $CuSO_4/K_2CrO_4$, hot and cold bath

D - $H_3BO_3/Na_2B_4O_7.10H_2O$, hot and cold bath

E - NaPCP in turpentine, cold soak

F - Bituminous paint (Pabco-Koppers Aust. Pty.Ltd.)

G - Untreated control

Trial Plan

3.5m

N

5.6m

A1	E1	D1	C1	F1	B1
E8	B2	F2	D2	E2	G1
F8	B3	A2	F7	E3	B4
C2	G2	C3	G3	D3	D8
E4	A4	F3	B5	A3	G4
C4	D4	F4	D5	G5	E7
B4	G6	D6	A5	C5	B7
E5	A6	C6	F5	D7	A8
G7	F6	G8	B8	E6	C8
A7	C7				

Eucalyptus obliqua sapwood stakes of 300 x 40 x 15mm dimensions were preservative-treated and emplaced at the Grove site for 36 weeks before examination.

Preservative Retentions

Treatment	A Creosote	B Tanalith C Paste	C $CuSO_4$/ K_2CrO_4	D H_3BO_3/ $Na_2B_4O_7.10H_2O$	E PCP	F Bituminous Paint
Preservative retention kg/m^3	45.0	NA	10.3	16.6	73.7	NA

NA = not applicable.

Increases in wood density after preservative treatment were used to calculate the preservative retentions. Each estimated retention was the mean of five determinations using wood blocks of 50 x 15 x 5mm diameter cut from the treated stakes.

Appendix 9 (continued)
Grove (Tasmania) Stake Trial - Data for Figure 17

Treatment	Stake No.	Cx-cellulase assay - % reduction viscosity NaCMC	Mycelial biomass - weight fungi (mg)/g wood	Fungal propagule count/0.01g sawdust	Predominant fungi (on cellulose agar)
HT Creosote	A1	34.0	nd	8	Fus.
	A2	33.7	45.2	0	-
	A3	22.2	nd	0	-
	A4	36.8	nd	0	-
	A5	32.5	nd	0	-
	A6	24.0	nd	0	-
	A7	29.5	84.1	0	-
	A8	17.0	79.2	0	-
Tanalith C Paste	B1	79.3	158.3	25	Trich., Phial.
	B2	43.0	nd	21	Trich., Phial.
	B3	82.6	19.8	8	Trich., Phial.
	B4	79.3	nd	2	Trich.
	B5	52.8	nd	1	Trich.
	B6	83.9	85.5	24	Trich., Phial.
	B7	34.1	70.1	10	Trich., Phial., Pen.
	B8	59.7	nd	29	Trich., Phial.
$CuSO_4/K_2CrO_4$	C1	74.5	475.0	29	Paec., Pen., Fus.
	C2	34.8	nd	14	Paec., Gon.
	C3	24.1	nd	37	Vert., Glio., Pen., Fus.
	C4	7.1	nd	28	Trich., Phial., Pen., Fus.
	C5	57.8	85.5	41	Phial., Pen., Vert., Fus.
	C6	22.7	nd	36	Vert., Phial., Pen., Fus.
	C7	10.6	252.3	23	Trich., Phial., Pen., Fus.
	C8	75.9	378.4	25	Trich., Pen., Fus.
$H_3BO_3/Na_2B_4O_7.10H_2O$	D1	55.1	nd	28	Glio., Phoma, Alt.
	D2	79.5	1187.5	51	Fus., Paec., Alt., Pen.
	D3	45.6	79.1	65	Phial., Pen., Fus.
	D4	67.5	475.0	53	Ulo., Alt., Vert., Fus.
	D5	83.0	356.3	33	Fus., Alt., Gon.
	D6	68.9	239.4	35	Phial., Fus., Ulo., Hum.
	D7	85.9	953.1	40	Alt., Ulo., Phial., Fus.
	D8	81.3	546.6	38	Hum., Pen., Alt., Gon.

Appendix 9 (Continued)

Grove (Tasmania) Stake Trial - Data for Figure 17 (Continued)

Treatment	Stake No.	Cx-cellulase assay - % reduction viscosity NaCMC	Mycelial biomass - weight fungi (mg)/g wood	Fungal propagule count/0.01g sawdust	Predominant fungi (on cellulose agar)
NaPCP	E1	20.3	nd	12	Ceph.
	E2	26.9	nd	22	Ceph.
	E3	25.6	nd	21	Phial., Ceph.
	E4	21.6	42.0	11	Fus., Trich.
	E5	18.3	119.7	17	Fus., Phial.
	E6	51.8	42.0	0	-
	E7	31.9	126.1	49	Trich., Oidio., Phial.
	E8	9.6	nd	5	Ceph.
Bituminous Paint	F1	79.7	98.1	23	Glio., Clad., Cord., Fus.
	F2	65.7	378.4	13	Gon., Pen., Hel.
	F3	66.4	153.9	17	Fus., Pen., Ceph.
	F4	47.2	119.7	25	Pen., Fus., Act.
	F5	14.3	39.6	21	Ceph., Amb., Fus., Pen.
	F6	28.0	nd	19	Pen., Fus., Trich., Ceph.
	F7	14.3	131.9	24	Vert., Ceph., Pen., Alt.
	F8	77.3	22.6	23	Fus., Mon., Pen.
Untreated (Control)	G1	47.2	99.0	29	Trich., Phial., Fus.
	G2	85.0	118.9	28	Trich., Graph., Phial.
	G3	81.1	1715.3	8	Trich., Pen., Mucor
	G4	76.0	475.0	6	Trich., Pen., Clad.
	G5	66.5	649.8	37	Trich., Pen., Gon.
	G6	78.0	826.7	32	Trich., Gon., Mucor

nd = not detectable.

The results shown correspond to individual Eucalyptus obliqua sapwood stakes (Stake A1, Stake A2, etc.). Stakes were emplaced for 36 weeks before analysis.

Abbreviations used:

Act. = Actinomycetes

Alt. = *Alternaria* sp.

Ceph. = *Cephalosporium acremonium*

Clad. = *Cladosporium herbarum*

Cord. = *Cordana* sp.

Fus. = *Fusarium* sp.

Glio. = *Gliocladium* sp.

Gon. = *Gonatobotrys* sp.

Hel. = *Helicoon* sp.

Hum. - *Humicola* sp.

Mon. = *Monilia* sp.

Mucor = *Mucor* sp.

Paec. = *Paecilomyces varioti*

Pen. = *Penicillium* spp.

Phial. = *Phialophora mutabilis*

Pyren. = *Pyrenochaeta* sp.

Trich. = *Trichoderma viride*

Ulo. = *Ulocladium* sp.

Vert. = *Verticillium* sp.

Enzyme assay values were obtained from 0.3g sawdust samples incubated in 10ml of 0.4% NaCMC (pH 5.5) for 1h at 50^{o}C.

Mycelial biomass estimates were based on chitin assay values assuming a mean 10% (w/w) chitin content of the fungal flora. Sawdust samples (0.2g) were hydrolysed in 5ml of 5N HCl for 20h at 80^{o}C.

Fungal propagule counts were obtained from 0.25% swollen cellulose agar plates, 0.01g sawdust per plate. The incubation period before counting was 12d at 22^{o}C.

Appendix 9 (continued)

Grove Stake Trial

Cx-cellulase Assay Data - Analysis of Variance

(for Figure 17)

Source of Variation	df	SS	MS	F
Treatment	6	17,347.4	2891.2	8.0 ($p<0.01$)
Error (within treatment)	47	16,957.9	360.8	
Total	53	34,305.3		

Chitin Assay Data - Analysis of Variance

Source of Variation	df	SS	MS	F
Treatment	6	11,695.62	1949.27	29.12 ($p<0.001$)
Error (within treatment)	47	3,146.01	66.94	
Total	53	14,841.63		

Fungal propagule count data (0.25% cellulose agar) - Analysis of Variance

Source of Variation	df	SS	MS	F
Treatment	6	7,995.5	1332.6	12.4 ($p<0.001$)
Error (within treatment)	47	5,029.8	107.2	
Total	53	13,025.3		

Appendix 10

Stability of Cx-cellulases after microbial death.

Leaching of Cx-cellulases from soft-rotted wood into water: Data for Figure 19.

	% Reduction in Viscosity NaCMC Days incubation at 22°C					
	0	15	30	45	60	75
Flask 1	0.0*	7.7	8.9*	7.6	9.5	7.9
Flask 2	0.0*	5.5	9.3	8.2	9.3	8.7
Flask 3	0.0*	6.9	9.6*	10.0	8.2	14.6
Flask 4	0.0*	6.3	7.6*	10.6	10.5	13.1

*5mg sodium azide added to stop microbial growth.

Flasks 1-4 contained sterile, soft-rotted Eucalyptus sp. wood blocks of 100 x 15 x 20mm approximate dimensions, positioned in 30g vermiculite saturated with 270ml water.

One ml aliquots of 'leachate' surrounding the soft-rotted wood blocks were added to 10ml of 0.5% NaCMC in 0.1M acetate buffer (pH 5.5) and incubated for 1h at 50°C.

Appendix 11

Analyses of soil samples from the Warrane (Tasmania) pole stub test site and the Grove (Tasmania) stake trial site.

	Warrane pole stub site Sample 1	2	3	4	Grove stake trial site
Total soluble salts (%)	0.02	0.04	0.02	0.02	0.13
Total Nitrogen (N) (parts per thousand)	0.53	0.67	0.56	0.56	3.4
Total Phosphorous (P) (parts per million)	140	225	75	163	906
Total Potassium (K) (parts per million)	100	120	80	70	250
pH	6.9	8.1	8.4	6.6	5.3

The above analyses were performed by the Tasmanian Government Analyst Laboratory, Hobart.

ABBREVIATIONS

ABBREVIATIONS

Å - Angstrom

BF3 - boron, fluorine-containing wood preservative

C - degree Centigrade

ca. - circa

C1 - exoglucanase

Cx - endoglucanase

CCA - copper-chrome-arsenic

CFB - copper-fluorine-boron based preservative

cm - centimetre

CMC - carboxymethyl cellulose

CSIRO - Commonwealth Scientific and Industrial Research Organisation

CSIRO/DBR - CSIRO Division of Building Research, Highett, Vic.

d - day(s)

df - degrees of freedom

F - variance ratio

Fig. - Figure

g - gram (or gravitational constant)

μg - microgram

h - hour

HT - High temperature (creosote)

J - joule

kg - kilogram

L (or l) - litre

LDS - Least significant difference

M - molar

m - metre

m^2 - $(metre)^2$

m^3 - $(metre)^3$

mA - milliampere

ml - millilitre

mm - millimetre

μm (or μ) - micron (10^{-6} metre)

mμ - millimicron (10^{-9} metre)

MBTH - 3-methyl-2-benzothiazolone hydrazone hydrochloride

min - minute

MS - mean square

NaCMC - sodium carboxymethyl cellulose

NaPCP - sodium pentachlorophenate

nm - nanometre (10^{-9} metre)

NSWFC - New South Wales Forestry Commission

P - probability

PCP - pentachlorophenol

PVC - polyvinyl chloride

r - correlation coefficient

(R) - registered trademark

RBBR - Remazol brilliant blue r dye

S1, S2, S3 - layers of the secondary wall of wood cells

SEM - scanning electron microscopy/microscope

sp(p). - species

SS - sum of squares

TLS - tangential longitudinal section

TS - transverse section

$\bar{x}$ - mean

XLPE - cross-linked polyethylene